PA NT COOPERATION TREAT

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 September 2000 (12.09.00)	
International application No. PCT/GB00/00511	**Applicant's or agent's file reference** P/23583.WO/ICB
International filing date (day/month/year) 15 February 2000 (15.02.00)	**Priority date** (day/month/year) 16 February 1999 (16.02.99)
Applicant DAVIS, Peter, David	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

03 August 2000 (03.08.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Pascal Piriou
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
P/23583.WO/ICB		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 00/00511	15/02/2000	16/02/1999
Applicant		
ANGIOGENE PHARMACEUTICALS LTD. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   COMBINATIONS FOR THE TREATMENT OF DISEASES INVOLVING ANGIOGENESIS

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

PATENT COOPERATION TREAT)

U 013589-7

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

BAILLIE, Iain C. et al.
LANGNER PARRY
52-54 High Holborn
London WC1V 6RR
GRANDE BRETAGNE

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	10.11.2000
Applicant's or agent's file reference: P/23583.WO/ICB	**REPLY DUE** **within 3 month(s)** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/00511	15/02/2000	16/02/1999

International Patent Classification (IPC) or both national classification and IPC

A61K31/198

Applicant

ANGIOGENE PHARMACEUTICALS LTD. et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☒	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain document cited
VII	☒	Certain defects in the international application
VIII	☒	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4. For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis. For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 16/06/2001.

Name and mailing address of the international preliminary examining authority:

European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer / Examiner

Pilling, S

Formalities officer (incl. extension of time limits)

Hundt, D

Telephone No. +49 89 2399 8042

Form PCT/IPEA/408 (cover sheet) (January 1994)

EXPRESS MAIL LABEL
NO.: EL 728214420 US

I. Basis of the opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".)*:

Description, pages:

1-9 as originally filed

Claims, No.:

1-14 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application,

☒ claims Nos. 12,13,

because:

☒ the said international application, or the said claims Nos. 12,13 relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-6,9-14
Inventive step (IS)	Claims	7,8
Industrial applicability (IA)	Claims	

2. Citations and explanations

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. Claims 12 and 13 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

2. The present application relates to compositions comprising a vascular damaging agent and inhibitors of the formation or action of nitric oxide (NO) for the treatment of diseases involving active angiogenesis (Claims 1 to 10); uses of a vascular damaging agent and a NO synthase inhibitor in the preparation of a medicament for the treatment of a disease involving active angiogenesis (Claim 11); methods of treating a disease involving active angiogenesis using a vascular damaging agent and a NO synthase inhibitor (Claims 12 and 13) and; uses of inhibitors of the formation or action of NO in the preparation of a medicament for augmenting the effects of a vascular damaging agent (Claim 14).

3. Claims 12 and 13 relate to methods of treatment of the human or animal body by therapy. In this regard, for the assessment of these claims with respect to industrial applicability, no unified criteria exist in the PCT. Furthermore, patentability can be dependent on the formulation of the claims. The EPO, for example does not recognize as industrially applicable, the subject matter of claims directed to a method of treatment of the human or animal body or to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

4. The documents cited in the International Search Report (ISR) are designated D1 to D8 as follows; if not indicated otherwise, reference is made to the passages

cited in said ISR.

D1: WO-A-9509621

D2: Br. J. Cancer (1998), 77(3), 426-433

D3: WO-A-9830537

D4: WO-A-9618617

D5: WO-A-9412165

D6: WO-A-9501972

D7: WO-A-9732585

D8: Seminars In Radiation Oncology, (1998) 8(3), 151-163

Claims 1 to 10; compositions comprising a vascular damaging agent and inhibitors of the formation or action of NO

5. Document D1 discloses pharmaceutical compositions comprising NO synthase inhibitors such as N^G-monomethyl-L-arginine or L-N-iminoethyl-ornithine in combination with cytokine-releasing anti-cancer agents such as 5,6-dimethylxanthenone acetic acid (DMX) and medical uses thereof

6. Furthermore document D2 discloses the combined pharmaceutical use of NO synthase inhibitors and DMX

7. Similarly documents D3 to D5 each show that the combination of NO synthase inhibitors with cytokine inducing agents such as DMX is further known.

8. Thus, the compositions defined in Claims 1 to 6 and Claims 9 and 10 is not new in view of the disclosures of each of documents D1 to D5 (Article 33(2) PCT).

9. None of the documents appears to disclose compositions comprising a NO synthase inhibitor in combination with a cytokine-releasing anti-cancer agent wherein the NO synthase inhibitor is a compound according to present Claim 7 or 8.

10. Thus, the subject matter of Claims 7 and 8 is new (Article 33(2) PCT).

11. Nevertheless, in view of the teaching in each of documents D1 to D5 that NO synthase inhibitors may be advantageously combined with DMX, there is considered to be no inventive step in merely selecting two further known NO synthase inhibitors (see documents D6 and D7) for combination with DMX wherein the new combination results in no surprising technical effect in comparison with the known combinations of NO inhibitors and DMX (see documents D1 to D5). Thus Claims 7 and 8 are considered to lack inventive step in view of the disclosures of any of documents D1 to D5 when considered in combination with either of documents D6 or D7 (Articles 33(3) PCT).

Claim 11; use of a vascular damaging agent and a NO synthase inhibitor in the preparation of a medicament for the treatment of a disease involving active angiogenesis

12. In addition to disclosing pharmaceutical compositions comprising NO synthase inhibitors in combination with cytokine-releasing anti-cancer agents, document D1 discloses their use to reduce tumour growth. The latter disease is presently said to involve active angiogenesis (see page 5 line 14 to 16).

13. Similarly document D2 describes the combined use of NO synthase inhibitors with DMX as anti-tumour agents

14. Thus, the subject matter of Claim 11 is not new in view of the disclosures of either of documents D1 or D2 (Article 33(2) PCT).

Claims 12 and 13; methods of treating diseases involving active angiogenesis using a vascular damaging agent and a NO synthase inhibitor

15. The comments set out herein above with respect to lack of novelty of Claim 11 are substantially applicable to the subject matter of Claims 12 and 13. With reference to the latter claim (13) it is noted that document D1 refers explicitly to concurrent administration of the NO synthase inhibitors and the cytokine-releasing anti-cancer agent. Thus, the subject matter of Claims 12 and 13 is not new in view of the disclosures of either of documents D1 or D2 (Article 33(2) PCT).

Claim 14; use of inhibitors of the formation or action of NO in the preparation of a medicament for augmenting the effects of a vascular damaging agent

16. The comments set out herein above with respect to lack of novelty of Claim 11 are substantially applicable to the subject matter of Claim 14. Thus, the subject matter of Claim 14 is not new in view of the disclosures of either of documents D1 or D2 (Article 33(2) PCT).

17. With reference to the observations set out herein above in respect of lack of novelty and inventive step of the present claims, it is recognized that the examples of the present application show that a synergistic anti-tumour effect can be obtained using combinations of particular NO synthase inhibitors (either L-NNA or AMP) with CA4P. In contrast none of the prior art documents appear to disclose or point towards the possibility of such a synergistic anti-tumoural effect. These prior art documents instead indicate that NO synthase inhibitors are useful in combination with DMX in order to avoid side effects associated with DMX induced production of NO. Nevertheless, the present discovery of a synergistic anti-tumoural effect is only relevant to patentability of the present claims in so far as it leads to a new and inventive product or process. No new or inventive product or process is presently defined by the claims of the application in suit.

Re Item VII
Certain defects in the international application

18. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in documents D1 and D2 is not mentioned in the description, nor are these documents identified therein.

Re Item VIII
Certain observations on the international application

19. The term "*vascular damaging agent*" used in Claims 1, 2, 11, 12 and 14 is vague and unclear thereby rendering the definition of the subject-matter of said claims unclear (Article 6 PCT). This definition does not define a recognised group of medicaments nor does the present description indicate how further "*vascular damaging agent*"(s) may be identified.

20. Furthermore, the term "*vascular damaging agent*" used in Claims 1, 2, 11, 12 and 14 is unduly broad and speculative (Article 6 PCT) since agents such as cholesterol would presently seem to be included within the scope of these claims but seem unlikely to find utility in the present invention.

21. Similarly the term "*inhibitor of the formation or action of nitric oxide*" in Claim 1 is also unduly broad and speculative (Article 6 PCT) in view of the disclosure only of inhibitors of nitric oxide synthase. Similar comments apply in respect of Claim 14.

22. The definition of "*an inhibitor of nitric oxide inhibitor*" in Claims 11 and 12 should apparently refer to "*an inhibitor of nitric oxide synthase*" (see page 2 lines 17 to 21).

23. Although Claims 1 and 2 have been drafted as separate independent claims, they appear to relate effectively to the same subject-matter and to differ from each other only with regard to the definition of the subject-matter for which protection is sought and in respect of the terminology used for the features of that subject-matter. The aforementioned claims therefore lack conciseness. Hence, Claims 1 and 2 do not meet the requirements of Article 6 PCT.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P/23583.WO/ICB		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/00511	15/02/2000	16/02/1999

International Patent Classification (IPC) or national classification and IPC
A61K31/198

Applicant
ANGIOGENE PHARMACEUTICALS LTD. et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
03/08/2000	01.06.2001

Name and mailing address of the international preliminary examining authority:

European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
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Authorized officer

Pilling, S

Telephone No. +49 89 2399 8461

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00511

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1,3-9	as originally filed			
2,2a	as received on	09/02/2001	with letter of	09/02/2000

Claims, No.:

9-15	as received on	09/02/2001	with letter of	09/02/2000
1-8	as received on	10/05/2001	with letter of	10/05/2001

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   ☐ the language of publication of the international application (under Rule 48.3(b)).
   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.
   ☐ filed together with the international application in computer readable form.
   ☐ furnished subsequently to this Authority in written form.
   ☐ furnished subsequently to this Authority in computer readable form.
   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:
   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 13,14.

because:

   ☒ the said international application, or the said claims Nos. 13,14 relate to the following subject matter which does not require an international preliminary examination (*specify*):
   see separate sheet

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-15
	No:	Claims
Inventive step (IS)	Yes:	Claims 1-15

	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-12,15 (for claims 13,14 see comments under Section V on separate sheet)
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. Claims 13 and 14 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

2. The present application relates to compositions comprising **a Vascular Damaging agent Other than a Cytokine Releasing agent** (VDOCR) and an inhibitor of the formation or action of **Nitric Oxide** (NO) for the treatment of diseases involving active angiogenesis (Claims 1 to 10); use of a VDOCR and an inhibitor of the formation or action of NO in the preparation of a medicament for the treatment of a disease involving active angiogenesis (Claims 11 and 12); methods of treating a disease involving active angiogenesis using a VDOCR and an inhibitor of the formation or action of NO (Claims 13 and 14) and; uses of inhibitors of the formation or action of NO in the preparation of a medicament for augmenting the effects of a VDOCR (Claim 15).

3. Claims 13 and 14 relate to methods of treatment of the human or animal body by therapy. In this regard, for the assessment of these claims with respect to industrial applicability, no unified criteria exist in the PCT. Furthermore, patentability can be dependent on the formulation of the claims. The EPO, for example does not recognize as industrially applicable, the subject matter of claims directed to a method of treatment of the human or animal body or to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

4. The following documents cited in the International Search Report (ISR) are

designated D1 to D5 as follows; if not indicated otherwise, reference is made to the passages cited in said ISR.

D1: WO-A-9509621

D2: Br. J. Cancer (1998), 77(3), 426-433

D3: WO-A-9830537

D4: WO-A-9618617

D5: WO-A-9412165

Claims 1 to 10; compositions comprising a VDOCR and an inhibitor of the formation or action of NO

4. None of the documents cited in the ISR disclose compositions comprising a vascular damaging agent other than a cytokine releasing agent (VDOCR) in combination with an inhibitor of the formation or action of nitric oxide (NO). Hence, the subject matter of Claims 1 to 10 is new (Article 33(2) PCT).

5. The closest prior art in respect of Claims 1 to 10 appears to be any of documents D1 to D5 that each disclose that NO synthase inhibitors may be usefully combined with cytokine releasing agents in order to ameliorate the inflammatory effects of the latter.

6. There would appear to be no reason, however, on the basis of any of documents D1 to D5 for the skilled man to have considered combining NO synthase inhibitors with vascular damaging agents other than cytokine releasing anticancer agents. In this regard, there is no suggestion in any of D1 to D5 that NO synthase inhibitors could be usefully combined with further active agents other than cytokine releasing agents. Moreover, the VDOCR defined in the present claims would not appear be directly inflammatory in the same way as the cytokine releasing anticancer agents of documents D1 to D5.

7. Moreover, the present Applicant has demonstrated a synergistic anti-tumour effect (see the present Examples) arising from the use of combinations of NO synthase inhibitors, *i.e.* L-NNA or AMP, with a combretastatin, *i.e.* CA4P. In contrast none of the prior art documents appear to disclose or point towards the possibility of such a synergistic anti-tumoural effect arising from the combination of VDOCR with an NO synthase inhibitor.

10. Thus, in view of the above considerations, it is considered that the subject matter of Claims 1 to 10 is inventive (Article 33(3) PCT).

Claims 11 to 15

8. For the reasons substantially as set out herein above in respect of Claims 1 to 10, it is also considered that the subject matter of Claims 11 to 15 is both novel and inventive (Article 33(2) and 33(3) PCT).

Re Item VIII
Certain observations on the international application

9. The definition of "*a nitric oxide synthase*" in Claim 12 should apparently rather refer to "*an inhibitor of nitric oxide synthase*" in accordance with originally filed page 2 lines 17 to 21 (Article 6 PCT).

10. The description has not yet been brought into agreement with the amended claims (Article 6 PCT).

growth is unclear and there have been reports of both tumour-stimulating and tumour-inhibiting effects (Chinje and Stratford, Essays Biochem. 32, 61-72, 1997). It has been suggested that the antitumour effects of 5,6-dimethylxanthenone acetic acid are mediated in part by nitric oxide production (Thompsen et al. Cancer Chemother Pharmacol. 31,151-5, 1992).

We have found that the efficacy of vascular damaging agents can be improved by combining the treatment with inhibitors of the formation or action of nitric oxide in a mammalian system.

In particular the efficacy of vascular damaging agents can be improved by combination with inhibitors of nitric oxide synthases, the enzymes that produce nitric oxide from arginine. In particular the efficacy of vascular damaging agents against tumours relatively resistant to their effects is improved by treatment with a nitric oxide synthase inhibitor.

Accordingly in one aspect of the invention we provide a method of treatment for a mammal having a disease that involves active angiogenesis such method comprising the administration of a therapeutic or subtherapeutic amount of a vascular damaging agent together with an inhibitor of nitric oxide synthase in an amount sufficient to augment the effect of the vascular damaging agent. The method is useful for the treatment of diseases such as cancers, especially solid tumours, psoriasis, diabetic retinopathy, macular degeneration, atherosclerosis and rheumatoid arthritis.

The vascular damaging agent and the nitric oxide synthase inhibitor can be administered together or separately. The method may be used as a sole therapy or in combination with other treatments. For the treatment of solid tumours compounds of the invention may be administered in combination with radiotherapy or in combination with other anti-tumour substances for example those selected from mitotic inhibitors, for example vinblastine, paclitaxel and docetaxel; alkylating agents, for example cisplatin, carboplatin and cyclophosphamide; antimetabolites, for example 5-fluorouracil, cytosine arabinoside and hydroxyurea; intercalating agents for example

CLAIMS:

1. A composition for the treatment of a disease involving active angiogenesis which comprises a vascular damaging agent together with an inhibitor of the formation or action of nitric oxide in a mammalian system.

2. A composition for the damage of the formation of new vasculature by angiogenesis comprising a combination of a vascular damaging agent and an amount of an inhibitor of nitric oxide synthase in an amount sufficient to augment the effect of the vascular damaging agent.

3. A composition according to claim 2 wherein said vascular damaging agent is selected from a tubulin-binding agent, a TNF-alpha inducing agent or an antibody targeted to vasculature.

4. A composition according to claims 2 and 3 wherein the nitric oxide synthase inhibitor is selected from a derivative of arginine, ornithine, lysine, citrulline, S-alkylthioureas or aminoguanidine.

5. A composition according to claim 4 wherein the nitric oxide synthase inhibitor is an N^G-substituted L-arginine selected from N^G-nitro–L-arginine and alkyl esters thereof, N^G-methyl-L-arginine and N^G-amino-L-arginine.

6. A composition according to claim 4 wherein the derivative of ornithine is L-N6-(1-iminoethyl)-ornithine.

7. A composition according to claim 4 wherein the derivative of lysine is L-N6-1-iminoethyl)-lysine.

8. A composition according to claim 4 wherein the derivative of citrulline is selected from L-thiocitrulline, L-homothiocitrulline or an S-alkylthiocitrulline particularly S-methyl-L-thiocitrulline.

9. A composition according to any one of claims 1 to 8 which comprises also a pharmaceutically acceptable excipent appropriate to the method of administration.

10. A composition according to any one of claims 1 to 9 wherein the composition is in the form of a kit, one part of the kit containing the vascular damaging agent and the second part of the kit the nitric oxide inhibitor.

11. Use in the preparation of a medicament for the treatment of disease involving active angiogenesis and containing a vascular damaging agent characterised in that the medicament also contains an amount of an inhibitor of nitric oxide inhibitor sufficient to augment the effect of the vascular damaging agent.

12. A method of treatment for a mammal having a disease involving active angiogenesis said method comprising administration of a vascular damaging agent and an amount of an inhibitor of nitric oxide inhibitor sufficient to augment the effect of the vascular damaging agent.

13. A method according to claim 12 wherein the vascular damaging agent and nitric oxide inhibitor are administered substantially simultaneously but separately to the mammal under treatment.

14. Use of inhibitors of nitric oxide formation or action in the preparation of a medicament for augmentation of the effects of a vascular damaging agent.

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9th February, 2001

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International Preliminary Examining Authority,
The European Patent Office,
D-80298 Munich,
GERMANY.

Dear Sir,

Re: Angiogene Pharmaceuticals Ltd. et al
PCT Application PCT/GB00/00511
Our Ref: P/23583.WO/ICB

In reply to the Written Opinion dated 10th November, 2000.

In regard to objection V and specifically in respect of statements 5 to 17, D1-D5 each deal with combination of DMXAA with NOS inhibitors. DMXAA is a cytokine releasing anticancer agent. The examiner makes the point that, although Applicant has shown a new aspect of the combinations, (the synergistic anti-tumour activity) the compositions with cytokine-releasing agents are themselves not new. Without prejudice Applicant withdraws cytokine-releasing agents from our claims. Since the DMXAA prior art is based largely on the anti-inflamatory activities of NOS inhibitors (ameliorating the effects of pro-inflammatory cytokines) it cannot be applied to the physiological/pharmacological observations which support the present invention. Therefore the combination with other vascular damaging agents, which have no pro-inflammatory activity, is new.

Specifically therefore the references to TNF-releasing agents have been excluded from the claims. The lack-of-novelty charge then has no basis whatsoever.

The same comments apply to former claim 13 onwards now 14 onwards.

We submit that the art has no suggestion that the combination of the present invention has a synergistic effect as now specified.

Continued.../

In regard to VII (18) D1 and D2 have been acknowledged in retyped page 2.

The Examiner in VIII (21) also mentioned that the effect has been demonstrated for "particular" NO synthase inhibitors, the implication being that we have not shown generality. Applicant in fact showed examples from two diverse structural groups of inhibitors, suggesting generality. It also generated data showing the effect for all nine of the inhibitors examined. These inhibitors are also from different structural groups and have different inhibitory profiles against the NOS isozymes and thus demonstrates the general nature of the effect conclusively.

Re: Item VIII

Paragraph 19

The term "vascular damaging agent" is well recognised in the art. It is clear that one skilled in the art would recognise that agents such as cholesterol would not come under this definition.

The term vascular damaging agent, as defined in the specification to be agents inducing damage in neovasculature, especially tumour vasculature, is widely understood and used by those skilled in the art of novel treatments for solid tumours. The term also has two often-used synonyms within the cancer community: vascular targeting agent and anti-vascular agent. Vascular damage is also sometimes referred to as vascular shutdown and is understood to mean a lasting reduction in the ability of the vasculature to act as a carrier of blood and nutrients. A search using the terms vascular damage (or damaging), vascular targeting, vascular shutdown and anti-vascular could identify all the prior art in this area and the term would not affect difficulty to one skilled in the art.

The biological tests for vascular damaging agents are widely agreed and well recognised. The most common is the test for reduction in perfused (functional) vascular volume within a tumour of an animal treated *in vivo* with the agent. This test is exemplified in the present case as well as in the art and can easily be reproduced by one skilled in the art. It therefore offers a method for recognising a vascular damaging agent.

Particular classes of compound are well known as vascular damaging agents. In particular the tubulin-binding compounds

Continued.../

colchicine, vinblastine, vincristine, combretastatin A4, combretastatin A4 phosphate, combretastatin A1, N-acetylcolchinol and its derivatives and the compounds known as AC-7700 and AC-7739 are described in the art to have this activity.

Paragraph 21

As we have previously discussed, the effects of these inhibitors are almost certainly mediated through a reduction in NO formation and subsequent action. A strong scientific rationale exists for the mechanism of these effects and it would definitely be expected from our results that any agent which prevents formation or action of NO would have the activity.

Paragraph 22

The Examiner is correct in that "an inhibitor of nitric oxide inhibitor" is a typographical error and is amended in the revised page of claims.

Paragraph 23

We submit that claim 2 which is limited to nitric oxide synthase is distinct in scope from claim 1 as well as the definition of the composition. We submit Applicant is entitled to protect the inventive concept by an independent claim to the most preferred "fall-back" position.

We submit that with the amendments now submitted namely

(1) Revised claims 1-15 to replace 1-14.
(2) Revised page 2 (now pages 2 and 2a)

We have distinguished from the art on terms of novelty and there is nothing in the cited art to suggest or render obvious the subject matter of the claims. The invention is also defined in a manner which would not create difficulties to those skilled in the art. All the terms used are conventional in this art.

Please acknowledge receipt of this letter and enclosures by return of the attached postal card.

Yours faithfully,

Enc.
ICB/LH

MARTYN W. MOLYNEAUX

CLAIMS:

1. A composition for the treatment of a disease involving active angiogenesis which comprises a vascular damaging agent other than a cytokine releasing anticancer agent together with an inhibitor of the formation or action of nitric oxide in a mammalian system.

2. A composition for the damage of the formation of new vasculature by angiogenesis comprising a combination of a vascular damaging agent other than a cytokine releasing anticancer agent and an amount of an inhibitor of nitric oxide synthase in an amount sufficient to augment the effect of the vascular damaging agent.

3. A composition according to claim 2 wherein said vascular damaging agent is selected from a tubulin-binding agent or an antibody targeted to vasculature.

4. A composition according to claims 2 and 3 wherein the nitric oxide synthase inhibitor is selected from a derivative of arginine, ornithine, lysine, citrulline, S-alkylthioureas or aminoguanidine.

5. A composition according to claim 4 wherein the nitric oxide synthase inhibitor is an N^G-substituted L-arginine selected from N^G-nitro–L-arginine and alkyl esters thereof, N^G-methyl-L-arginine and N^G-amino-L-arginine.

6. A composition according to claim 4 wherein the derivative of ornithine is L-N6-(1-iminoethyl)-ornithine.

7. A composition according to claim 4 wherein the derivative of lysine is L-N6-1-iminoethyl)-lysine.

8. A composition according to claim 4 wherein the derivative of citrulline is selected from L-thiocitrulline, L-homothiocitrulline or an S-alkylthiocitrulline particularly S-methyl-L-thiocitrulline.

9. A composition according to any one of claims 1 to 8 which also comprises a pharmaceutically acceptable excipent appropriate to the method of administration.

10. A composition according to any one of claims 1 to 9 wherein the composition is in the form of a kit, one part of the kit containing the vascular damaging agent and the second part of the kit the nitric oxide inhibitor.

11. Use in the preparation of a medicament for the treatment of disease involving active angiogenesis and containing a vascular damaging agent other than a cytokine releasing anticancer agent characterised in that the medicament also contains an amount of an inhibitor of formation or action of nitric oxide sufficient to augment the effect of the vascular damaging agent.

12. Use according to claim 11 wherein said inhibitor of formation or action of nitric oxide is a nitric oxide synthase.

13. A method of treatment for a mammal having a disease involving active angiogenesis said method comprising administration of a vascular damaging agent other than a cytokine releasing anti cancer agent and an amount of an inhibitor of formation or action of nitric oxide in amount sufficient to augment the effect of the vascular damaging agent.

14. A method according to claim 13 wherein the vascular damaging agent and nitric oxide inhibitor are administered substantially simultaneously but separately to the mammal under treatment.

15. Use of inhibitors of nitric oxide formation or action in the preparation of a medicament for augmentation of the effects of a vascular damaging agent other than a cytokine releasing agent.

growth is unclear and there have been reports of both tumour-stimulating and tumour-inhibiting effects (Chinje and Stratford, Essays Biochem. 32, 61-72, 1997). It has been suggested that the antitumour effects of 5,6-dimethylxanthenone acetic acid are mediated in part by nitric oxide production (Thompsen et al. Cancer Chemother Pharmacol. 31,151-5, 1992).

WO-A 9509621 and Br. J Cancer (1998), 77(3), 426-433 disclose combinations of cytokine releasing anticancer agents (TNF-releasing agents). These relate to ameliorating the effects of pro-inflammatory cytokines. There is no suggestion of synergistic activity from a combination of a vascular damaging agent (many of which have no pro-inflammatory activity) and an NO inhibitor.

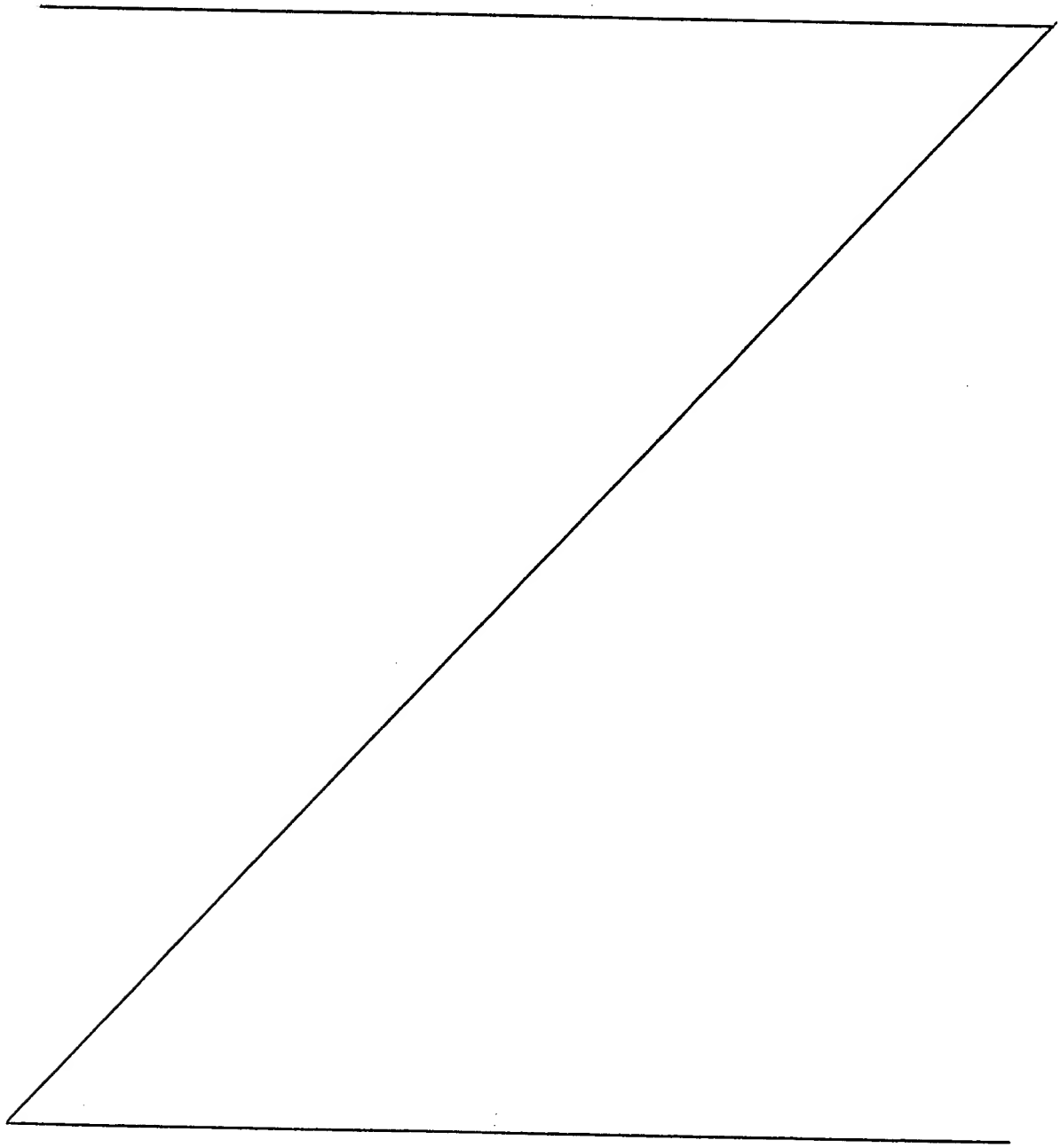
We have found that the efficacy of vascular damaging agents can be improved by combining the treatment with inhibitors of the formation or action of nitric oxide in a mammalian system.

In particular the efficacy of vascular damaging agents can be improved by combination with inhibitors of nitric oxide synthases, the enzymes that produce nitric oxide from arginine. In particular the efficacy of vascular damaging agents against tumours relatively resistant to their effects is improved by treatment with a nitric oxide synthase inhibitor.

Accordingly in one aspect of the invention we provide a method of treatment for a mammal having a disease that involves active angiogenesis such method comprising the administration of a therapeutic or subtherapeutic amount of a vascular damaging agent together with an inhibitor of nitric oxide synthase in an amount sufficient to augment the effect of the vascular damaging agent. The method is useful for the treatment of diseases such as cancers, especially solid tumours, psoriasis, diabetic retinopathy, macular degeneration, atherosclerosis and rheumatoid arthritis.

The vascular damaging agent and the nitric oxide synthase inhibitor can be administered together or separately. The method may be used as a sole therapy or in

combination with other treatments. For the treatment of solid tumours compounds of the invention may be administered in combination with radiotherapy or in combination with other anti-tumour substances for example those selected from mitotic inhibitors, for example vinblastine, paclitaxel and docetaxel; alkylating agents, for example cisplatin, carboplatin and cyclophosphamide; antimetabolites, for example 5-fluorouracil, cytosine arabinoside and hydroxyurea; intercalating agents for example



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(54) Title: COMBINATIONS FOR THE TREATMENT OF DISEASES INVOLVING ANGIOGENESIS

(57) Abstract

Compositions for the inhibition of the formation of new vasculature by angiogenesis are provided comprising the combination of a vasculature damaging agent and an inhibitor of the formation or action of nitric oxide in mammalian systems. There are also provided the use of said combinations in medicaments and kits of said compounds and treatment employing said materials.

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COMBINATIONS FOR THE TREATMENT OF DISEASES INVOLVING ANGIOGENESIS

This invention relates to a method for treating diseases involving active angiogenesis, to compositions useful for the treatment of diseases involving angiogenesis and to the use of the compositions in the preparation of a medicament for the treatment of diseases involving active angiogenesis. In one aspect of the invention the method involves the administration to a mammal of an inhibitor of nitric oxide in combination with a compound inducing vascular damage.

Formation of new vasculature by angiogenesis is a key pathological feature of several diseases (J Folkman, New England Journal of Medicine 333, 1757-1763, 1995). For example, for a solid tumour to grow it must develop its own blood supply upon which it depends critically for the provision of oxygen and nutrients; if this blood supply is mechanically shut off the tumour undergoes necrotic death. Neovascularisation is also a clinical feature of skin lesions in psoriasis, of the invasive pannus in the joints of rheumatoid arthritis patients and of atherosclerotic plaques. Retinal neovascularisation is pathological in macular degeneration and in diabetic retinopathy. In all these diseases reversal of neovascularisation by damaging the newly-formed vascular endothelium is expected to have a beneficial therapeutic effect.

Certain chemical compounds have been shown to have vascular damaging activity against the newly formed endothelium of solid tumours. These agents include, for example, combretastatin A4 phosphate (Dark et al., Cancer Research 57, 1829-1834, 1997), combretastain analogues (for example those described in J Med Chem 41, 3022-32,1998 by Ohsumi et al.), the flavone acetic acids, for example 5,6-dimethylxanthenone acetic acid (Zwi, Pathology, 26, 161-9, 1994), colchicine (Baguley et al. Eur J Cancer 27, 482-7, 1991). However some tumours are resistant to these agents.

One characteristic of tumours relatively resistant to vascular damaging agents is their ability to produce large amounts of nitric oxide. The role of nitric oxide in tumour

growth is unclear and there have been reports of both tumour-stimulating and tumour-inhibiting effects (Chinje and Stratford, Essays Biochem. 32, 61-72, 1997). It has been suggested that the antitumour effects of 5,6-dimethylxanthenone acetic acid are mediated in part by nitric oxide production (Thompsen et al. Cancer Chemother Pharmacol. 31,151-5, 1992).

We have found that the efficacy of vascular damaging agents can be improved by combining the treatment with inhibitors of the formation or action of nitric oxide in a mammalian system.

In particular the efficacy of vascular damaging agents can be improved by combination with inhibitors of nitric oxide synthases, the enzymes that produce nitric oxide from arginine. In particular the efficacy of vascular damaging agents against tumours relatively resistant to their effects is improved by treatment with a nitric oxide synthase inhibitor.

Accordingly in one aspect of the invention we provide a method of treatment for a mammal having a disease that involves active angiogenesis such method comprising the administration of a therapeutic or subtherapeutic amount of a vascular damaging agent together with an inhibitor of nitric oxide synthase in an amount sufficient to augment the effect of the vascular damaging agent. The method is useful for the treatment of diseases such as cancers, especially solid tumours, psoriasis, diabetic retinopathy, macular degeneration, atherosclerosis and rheumatoid arthritis.

The vascular damaging agent and the nitric oxide synthase inhibitor can be administered together or separately. The method may be used as a sole therapy or in combination with other treatments. For the treatment of solid tumours compounds of the invention may be administered in combination with radiotherapy or in combination with other anti-tumour substances for example those selected from mitotic inhibitors, for example vinblastine, paclitaxel and docetaxel; alkylating agents, for example cisplatin, carboplatin and cyclophosphamide; antimetabolites, for example 5-fluorouracil, cytosine arabinoside and hydroxyurea; intercalating agents for example

adriamycin and bleomycin; enzymes, for example aspariginase; topoisomerase inhibitors for example etoposide, topotecan and irinotecan; thymidylate synthase inhibitors for example raltitrexed; biological response modifiers for example interferon; antibodies for example edrecolomab; and anti-hormones for example tamoxifen. Such combination treatment may involve simultaneous or sequential application of the individual components of the treatment.

The vascular damaging agent and the nitric oxide synthase inhibitor can be administered by the same route or by different routes. Such routes of administration include oral, buccal, nasal, topical, rectal and parenteral administration. Each component of the method, the vascular damaging agent and the nitric oxide synthase inhibitor may independently be administered in a form suitable for the intended route of administration and such forms may be prepared in a conventional manner using conventional excipients. For example for oral administration the pharmaceutical compositions may take the form of tablets or capsules. For nasal administration or administration by inhalation the compounds may be conveniently delivered as a powder or in solution. Topical administration may be as an ointment or cream and rectal administration may be as a suppository. For parenteral injection (including intravenous, subcutaneous, intramuscular, intravascular or infusion) the composition may take the form of, for example, a sterile solution, suspension or emulsion.
The preferred route of administration of each component will depend on the disease being treated. For solid tumours the components may each advantageously be delivered, either together or separately, as an intravenous infusion.

Vascular damaging agents are compounds which induce selective damage to newly formed, rather than established, vasculature. Many such compounds are known and it is considered this invention is generally applicable to such agents. Such agents include tubulin-binding agents, for example the combretastatins and their prodrugs, the colchinols and their prodrugs and (Z)-2-methoxy-5-[2-(3,4,5-trimethoxyphenyl)vinyl]phenylamine and its prodrugs, TNF-alpha inducing agents such as the xanthenone acetic acids, for example dimethylxanthenoneacetic acid, and antibodies targeted to the vasculature.

A wide variety of compounds which inhibit the formation or action of nitric oxide in mammalian systems can be employed. Specifically nitric oxide synthase inhibitors are those compounds which inhibit any of the forms of nitric oxide synthase. Such agents include derivatives of arginine, ornithine, lysine and citrulline, S-alkylthioureas and aminoguanidines. Where the nitric oxide synthase inhibitor is a derivative of arginine it may be, for example, an N^G-substituted L-arginine selected from N^G-nitro-L-arginine and alkyl esters thereof, N^G-methyl-L-arginine and N^G-amino-L-arginine. Where the nitric oxide synthase inhibitor is a derivative of ornithine it may be, for example L-N6-(1-iminoethyl)-ornithine. Where the nitric oxide synthase inhibitor is a derivative of lysine it may be, for example L-N6-(1-iminoethyl)-lysine. Where the nitric oxide synthase inhibitor is a derivative of citrulline it may be, for example L-thiocitrulline, L-homothiocitrulline or an S-alkylthiocitrulline such as S-methyl-L-thiocitrulline.

In a further embodiment of the invention there is provided a composition for the treatment of diseases involving active angiogenesis. The composition of the invention comprises a vascular damaging agent in combination with a nitric oxide synthase inhibitor where both the vascular damaging agent and the nitric oxide synthase inhibitor are as hereinbefore defined.

Thus for example the composition may contain for example a combretastatin derivative, a colchicine derivative, a colchinol derivative, a xanthenone acetic acid derivative or a vascular targeted antibody, in combination with a nitric oxide synthase inhibitor for example a derivative of arginine, a derivative of ornithine, a derivative of lysine, a derivative of citrulline, a S-alkylthioureas or an aminoguanidine.

Particular examples of vascular damaging agents that may be present in the composition include combretastatin A4 and its prodrugs for example combretastatin A4 phosphate, (Z)-2-methoxy-5-[2-(3,4,5-trimethoxyphenyl)vinyl]phenylamine and its prodrugs, N-acetylcolchinol and its prodrugs for example N-acetylcolchinol-O-phosphate and 5,6-dimethylxanthenoneacetic acid.

Particular examples of nitric oxide synthase inhibitors which may be present in the composition include derivatives of arginine, ornithine, lysine and citrulline, S-alkylthioureas aminoguanidines and aminopyridines. Where the nitric oxide synthase inhibitor is a derivative of arginine it may be, for example, an N^G-substituted L-arginine selected from N^G-nitro-L-arginine and alkyl esters thereof, N^G-methyl-L-arginine and N^G-amino-L-arginine. Where the nitric oxide synthase inhibitor is a derivative of ornithine it may be, for example L-N6-(1-iminoethyl)-ornithine. Where the nitric oxide synthase inhibitor is a derivative of lysine it may be, for example L-N6-(1-iminoethyl)-lysine. Where the nitric oxide synthase inhibitor is a derivative of citrulline it may be, for example L-thiocitrulline, L-homothiocitrulline or an S-alkylthiocitrulline such as S-methyl-L-thiocitrulline. Where the nitric oxide synthase inhibitor is an aminopyridine it may be for example 2-amino-4-methylpyridine.

The composition is useful for the treatment of diseases involving active angiogenesis for example solid tumours, psoriasis, diabetic retinopathy, macular degeneration, atherosclerosis and rheumatoid arthritis.

The relative proportion of each component will be determined by the identity of each individual vascular damaging agent or nitric oxide synthase inhibitor and by the disease to be treated.

The composition may include pharmaceutically acceptable excipients selected with regard to the intended route of administration and standard pharmaceutical practice. The composition may take a form suitable for oral, buccal, nasal, topical, rectal or parenteral administration and may be prepared in a conventional manner using conventional excipients. For example for oral administration the composition may take the form of tablets or capsules. For nasal administration or administration by inhalation the compounds may be conveniently delivered as a powder or in solution. Topical administration may be as an ointment or cream and rectal administration may be as a suppository. For parenteral injection (including intravenous, subcutaneous, intramuscular, intravascular or infusion) the composition may take the form of, for example, a sterile solution, suspension or emulsion.

The dose of a compound of the invention required for the prophylaxis or treatment of a particular condition will vary depending on the identity of the individual components, the route of administration, the form and severity of the condition and whether the compound is to be administered alone or in combination with another drug. Thus the precise dose will be determined by the administering physician and will depend on the particular vascular damaging agent and NO synthase inhibitor in the composition. However the dose of the vascular damaging agent envisaged is, for example, in the range 10-1000mg/m^2 body surface, preferably 20-200mg/ m^2 and that for the nitric oxide inhibitor 1-1000mg/m^2, preferably 5-500mg/ m^2. A unit dose form of the vascular damaging agent as, for example, a sterile solution for injection will usually contain, for example, 40-400mg of the active ingredient. A unit dose form of the nitric oxide synthase inhibitor as, for example, a sterile solution for injection will usually contain, for example, 10-1000mg of the active ingredient. A unit dose form of a composition containing both a vascular damaging agent and a nitric oxide synthase inhibitor as , for example, a sterile solution for injection will usually contain, for example, 40-400mg of the vascular damaging agent and 10-1000mg of the nitric oxide synthase inhibitor.

The composition of the invention may be administered as a sole therapy or in combination with other treatments. For the treatment of solid tumours the composition may be administered in combination with radiotherapy or in combination with other anti-tumour substances for example those selected from mitotic inhibitors, for example vinblastine, paclitaxel and docetaxel; alkylating agents, for example cisplatin, carboplatin and cyclophosphamide; antimetabolites, for example 5-fluorouracil, cytosine arabinoside and hydroxyurea; intercalating agents for example adriamycin and bleomycin; enzymes, for example aspariginase; topoisomerase inhibitors for example etoposide, topotecan and irinotecan; thymidylate synthase inhibitors for example raltitrexed; biological response modifiers for example interferon; antibodies for example edrecolomab; and anti-hormones for example tamoxifen. Such combination treatment may involve simultaneous or sequential application of the individual components of the treatment.

In a further embodiment of the invention we provide the use of a compostion of the invention for the preparation of a medicament for the treatment of a disease involving active angiogenesis.

The invention will now be illustrated by the following Examples in which biological assays are used to illustrate the invention:

Induction of necrosis

Mice bearing either CaNT or SaS tumours were treated with the test compound and tumours excised after 24h, fixed in formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin. Sections were scored based on area of necrosis as follows:

% necrosis	score		% necrosis	score
0-10	1		51-60	6
11-20	2		61-70	7
21-30	3		71-80	8
31-40	4		81-90	9
41-50	5		91-100	10

Control tumours had mean scores of 2.0 (CaNT) and 1.0 (SaS).

EXAMPLE 1

In this assay the effect of a given dose of either a vascular damaging agent or a nitric oxide synthase inhibitor administered alone can be compared with the effect of a combination of the two agents.

Table 1: Enhancement of Combretastatin A4 phosphate (CA4P) activity in SaS tumours by coadministration of L-N^G-nitroarginine (L-NNA)

Treatment	Necrosis score ±SEM (n)
None	1.0±0 (10)
CA4P, 500mg/kg	1.7±0.7 (3)
L-NNA, 10mg/kg	2.0±1 (3)
CA4P, 500mg/kg + L-NNA 10mg/kg	9.0±0 (3)

EXAMPLE 2

Table 2: Enhancement of Combretastatin A4 phosphate (CA4P) activity in SaS tumours by coadministration of 2-amino-4-methylpyridine (AMP)

Treatment	Necrosis score ±SEM (n)
None	1.0±0 (10)
CA4P, 500mg/kg	1.7±0.7 (3)
AMP, 10mg/kg	1.0 (2)
CA4P, 500mg/kg + AMP 10mg/kg	4.5 (2)

EXAMPLE 3

Activity against tumour vasculature measured by fluorescent dye.

The following experiment further demonstrates the ability of the compounds to damage tumour vasculature.

Tumour functional vascular volume in CaNT tumour-bearing mice was measured using the fluorescent dye Hoechst 33342 according to the method of Smith *et al* (Brit J Cancer 57, 247-253, 1988). The fluorescent dye was dissolved in saline at 6.25 mg/ml and injected intravenously at 10 mg/kg 24 hours after intra peritoneal drug treatment. One minute later, animals were killed and tumours excised and frozen; 10 μm sections were cut at 3 different levels and observed under UV illumination using an Olympus microscope equipped with epifluorescence. Blood vessels were identified by their fluorescent outlines and vascular volume was quantified using a point scoring system based on that described by Chalkley, (J Natl Cancer Inst, 4, 47-53, 1943). All estimates were based on counting a minimum of 100 fields from sections cut at the 3 different levels.

Table 3: Enhancement of Combretastatin A4 phosphate (CA4P) activity in CaNT tumours by coadministration of L-N^G-nitroarginine (L-NNA).

Treatment	Vascular Volume % ±SEM (n)
None	2.35
CA4P, 25mg/kg	1.03±0.14 (4)
L-NNA, 10mg/kg	2.45±0.04 (3)
CA4P, 25mg/kg + L-NNA 10mg/kg	0.63±0.25 (3)

CLAIMS:

1. A composition for the treatment of a disease involving active angiogenesis which comprises a vascular damaging agent together with an inhibitor of the formation or action of nitric oxide in a mammalian system.

2. A composition for the damage of the formation of new vasculature by angiogenesis comprising a combination of a vascular damaging agent and an amount of an inhibitor of nitric oxide synthase in an amount sufficient to augment the effect of the vascular damaging agent.

3. A composition according to claim 2 wherein said vascular damaging agent is selected from a tubulin-binding agent, a TNF-alpha inducing agent or an antibody targeted to vasculature.

4. A composition according to claims 2 and 3 wherein the nitric oxide synthase inhibitor is selected from a derivative of arginine, ornithine, lysine, citrulline, S-alkylthioureas or aminoguanidine.

5. A composition according to claim 4 wherein the nitric oxide synthase inhibitor is an N^G-substituted L-arginine selected from N^G-nitro–L-arginine and alkyl esters thereof, N^G-methyl-L-arginine and N^G-amino-L-arginine.

6. A composition according to claim 4 wherein the derivative of ornithine is L-N6-(1-iminoethyl)-ornithine.

7. A composition according to claim 4 wherein the derivative of lysine is L-N6-1-iminoethyl)-lysine.

8. A composition according to claim 4 wherein the derivative of citrulline is selected from L-thiocitrulline, L-homothiocitrulline or an S-alkylthiocitrulline particularly S-methyl-L-thiocitrulline.

9. A composition according to any one of claims 1 to 8 which comprises also a pharmaceutically acceptable excipent appropriate to the method of administration.

10. A composition according to any one of claims 1 to 9 wherein the composition is in the form of a kit, one part of the kit containing the vascular damaging agent and the second part of the kit the nitric oxide inhibitor.

11. Use in the preparation of a medicament for the treatment of disease involving active angiogenesis and containing a vascular damaging agent characterised in that the medicament also contains an amount of an inhibitor of nitric oxide inhibitor sufficient to augment the effect of the vascular damaging agent.

12. A method of treatment for a mammal having a disease involving active angiogenesis said method comprising administration of a vascular damaging agent and an amount of an inhibitor of nitric oxide inhibitor sufficient to augment the effect of the vascular damaging agent.

13. A method according to claim 12 wherein the vascular damaging agent and nitric oxide inhibitor are administered substantially simultaneously but separately to the mammal under treatment.

14. Use of inhibitors of nitric oxide formation or action in the preparation of a medicament for augmentation of the effects of a vascular damaging agent.

INTERNATIONAL SEARCH REPORT

Int Ional Application No
PCT/GB 00/00511

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/198 A61K31/44 A61K31/66 A61P35/00 A61P17/06
A61P27/00 A61P9/10 A61P19/02 A61K45/06 //(A61K31/44, 31:195),(A61K31/66,31:195)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 09621 A (WELLCOME FOUND ;THOMSEN LINDY LOUISE (GB); KNOWLES RICHARD GRAHAM) 13 April 1995 (1995-04-13)	1-14
Y	page 2, line 9 -page 2, line 11 page 2, line 22 -page 2, line 26 claims 1,6,7	7,8
X	MOILANEN, E. ET AL: "Persistent induction of nitric oxide synthase in tumors from mice treated with the anti-tumor agent 5,6-dimethylxanthenone-4-acetic acid" BR. J. CANCER , vol. 77, no. 3, 1988, pages 426-433, XP000885438	1-14
Y	page 432, column 1, line 21 -page 432, column 2, line 2	7,8

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 May 2000	25. 05. 00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Pilling, S

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 30537 A (BEAMS RICHARD MANSFIELD ;DRYSDALE MARTIN JAMES (GB); HODSON HAROLD) 16 July 1998 (1998-07-16)	1-10,14
Y	abstract page 3, line 33 -page 4, line 2	7,8
X	WO 96 18617 A (MERCK & CO INC ;GUTHIKONDA RAVINDRA K (US); HAGMANN WILLIAM K (US)) 20 June 1996 (1996-06-20)	1-10,14
Y	abstract page 4, line 22 -page 4, line 26	7,8
X	WO 94 12165 A (WELLCOME FOUND ;GARVEY EDWARD PATRICK (US); TANOURY GERALD JOSEPH) 9 June 1994 (1994-06-09)	1-10,14
Y	abstract page 3, line 16 -page 3, line 20	7,8
Y	WO 95 01972 A (WISCONSIN MED COLLEGE INC) 19 January 1995 (1995-01-19) page 2, line 15 -page 2, line 19 page 7, line 21 -page 12, line 9	8
Y	WO 97 32585 A (LAI CHING SAN ;MEDINOX INC (US)) 12 September 1997 (1997-09-12) claim 16	7
A	CHAPLIN D.J. ET AL: "Modification of tumor blood flow: Current status and future directions." SEMINARS IN RADIATION ONCOLOGY, vol. 8, no. 3, 1998, page 151-163 XP000885557 the whole document	1-14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 00/00511

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-14 in part
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-14 in part

The present claims relate to compositions, uses and methods involving a combination of one group of functionally defined active agents with a further group of functionally defined active agents. Evidently each these functionally defined groups of compounds includes a large number of possible compounds resulting in many thousands of possible combinations.

It is further noted that in general the definition of compounds in terms of functional parameters makes a complete search impossible. In this regard, it is not always disclosed in the searched prior art documents whether candidate compound(s) would fulfill the requirements of such functional parameters or not.

Furthermore, in the present case it is considered that the definition of the "vascular damaging agent" as used in the claims is particularly unclear. This definition does not relate to a commonly recognized group of compounds nor does the description set out any clear objective way in which such agents may be identified. Similarly with reference to the definitions of the "inhibitor of the formation or action of nitric oxide" (see Claim 1) and "inhibitor of nitric oxide inhibitor" (see Claims 11 and 12), it is noted that the present specification only provides support for particular nitric oxide synthase inhibitors.

Consequently, the search has been carried out for those parts of the application which do appear to be clear and supported by the present description and for which a search is feasible, namely combinations of (i) the particular vascular damaging agents which are identified in the description (see page 3 lines 25 to 32, page 4 lines 27 to 31 and the examples) with (ii) the particular nitric oxide synthase inhibitors which are identified in the description (see page 4 lines 1 to 13, page 5 lines 1 to 12 and the examples) and the general concept/idea underlying the invention.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/00511

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9509621 A	13-04-1995	AU 7787694 A	01-05-1995
		ZA 9407754 A	04-04-1996
WO 9830537 A	16-07-1998	AU 6208398 A	03-08-1998
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		CN 1095710 A	30-11-1994
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		CA 2166222 A	19-01-1995
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		JP 8512318 T	24-12-1996
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		US 5663364 A	02-09-1997
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		AU 6998498 A	30-07-1998
		CA 2238029 A	12-09-1997

EXPERIMENTAL CELL RESEARCH 218, 189–200 (1995)

Colchicine Induces Apoptosis in Cerebellar Granule Cells

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Exposure to 1 μM colchicine, a microtubule disrupting agent, triggered apoptosis in rat cerebellar granule cells (CGC). Apoptotic nuclei began to appear after 12 h followed by oligonucleosomal DNA laddering, whereas inhibition of the mitochondrial 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide metabolism became significant between 18 and 24 h, when most cells already had apoptotic nuclei. These events were preceded by loss of tau protein and fragmentation of α and β tubulins. Colchicine treatment also caused alterations in Ca^{2+} responses to chemical depolarization and a moderate, but progressive, increase in the resting intracellular Ca^{2+} concentration. Nearly all neurons expressed c-Fos after the treatment with colchicine. However, while in part of the cell population c-Fos levels subsequently declined, in the neurons undergoing apoptosis the protein was still expressed, but had an abnormal intracellular localization. An increased expression of the constitutive nitric oxide synthase (NOS-I) was also detected at 12 h and was followed by increased nitrite production. Treatment with 100 nM taxol to stabilize the microtubuli prevented DNA laddering and apoptotic body formation induced by colchicine. In contrast, pretreatment with the N-methyl-D-aspartate receptor-antagonist, MK-801, or L-type Ca^{2+} channel blockers did not prevent colchicine-induced CGC apoptosis. Inhibitors of NOS were also ineffective in preventing apoptotic body formation and DNA laddering, whereas they delayed the secondary cell lysis. These results support the idea that colchicine-induced cytoskeletal alterations directly initiate the genetic and structural modifications that result in CGC apoptosis.

INTRODUCTION

Abnormalities in the cytoskeleton are a frequent component of several neurological disorders [1]. For example, loss of tau (a low-molecular-weight, microtubule-associated protein) from axons and the accumulation of phosphorylated tau in neural cell bodies have been associated with the formation of neurofibrillary tangles, a histopathological feature of Alzheimer's disease [2–5]. Cytoskeletal elements with altered immunoreactivity (i.e., tau and other microtubule-associated proteins) are also abundant in the neurofibrillary tangles found in normal aged brains or in neurodegenerative disorders besides Alzheimer's disease [6, 7]. Interestingly, antigenic changes similar to those found in neurofibrillary tangles can also be reproduced *in vitro* by exposing cultured hippocampal neurons to glutamate, Ca^{2+} ionophore, or high extracellular K^+ [8].

While the events responsible for the cytoskeletal alterations still remain unclear, several observations suggest that cytoskeletal damage can result in neural cell death. For example, microtubule stabilization by taxol prevents the changes in tau immunoreactivity [9] and attenuates glutamate-induced neural toxicity [10]. Findings that the amyloid β-protein activates apoptosis in embryonic rat hippocampal neurons following an increase in tau protein kinase I activity and tau phosphorylation also suggest a role for cytoskeletal abnormalities in the development of neural cell death [11]. Enhanced tau phosphorylation results in changes in the protein immunoreactivity properties, as observed in neurofibrillary tangles [9].

Cytoskeletal alterations similar to those observed in neurofibrillary tangles are also elicited by Ca^{2+} overload [8]. The latter may contribute to the onset of apoptosis in neural cells exposed to β-amyloid fragments [12, 13] or after glutamate overstimulation [14, 15]. These findings suggest that an initial disturbance in Ca^{2+} homeostasis would—at least in some systems—cause both the cytoskeletal alterations and the loss of neural cells observed in several neurodegenerative disorders. On the other hand, primary cytoskeletal alterations can promote ion movements that can result in changes in intracellular Ca^{2+} concentration or alter Ca^{2+} responses to agonists [16–18]. Thus, the two mechanisms may not be mutually exclusive, but may potentiate one another.

In view of these considerations, we designed this study to investigate the effects of a primary cytoskeletal damage on the survival of cerebellar granule cells (CGC). Among several cytoskeletal agents, we selected colchi-

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0014-4827/95 $6.00

cine, a microtubule disrupting drug that also promotes tau protein modifications. Colchicine binds to and depolymerizes the microtubules [19], and exposure to colchicine is known to increase the immunoreactivity of neurons toward tau antibodies specific for the neurofibrillary tangles of the Alzheimer's disease [9]. In addition, *in vivo* treatment with colchicine causes neurofibrillary degeneration [20] and neural cell loss [21]. Because of these properties colchicine has been used extensively as a model to mimic *in vitro* some cytoskeletal alterations found in neurodegenerative diseases [22].

MATERIALS AND METHODS

Materials. Colchicine, Nω-nitro-L-arginine, *N*-methyl-monoarginine (NMMA), taxol, poly-L-lysine (M_r > 300.000), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO). Proteinase K and primary mouse monoclonal and rabbit polyclonal antibodies raised against tau and tubulins, respectively, were obtained from Boehringer-Mannheim (Germany). Fura-2/AM and fluo-3/AM were purchased from Molecular Probes (Eugene, OR). Agarose was from FMC BioProducts. The NMDA antagonist, (+)-methyl-10,11-dihydro-5H-benzo-(a,d) cyclohepten-5,10-imine hydrogen maleate (MK-801) was from RBI Research Biochemicals International (Natick, MA). All other chemicals were of the highest grade of purity. Compounds were prepared as stocks in phosphate-buffered saline (PBS) or dimethyl sulfoxide and appropriate amounts of the solvent were added to control cultures. Cell culture media and supplements were obtained from Gibco.

Cell culture and treatments. CGC were prepared from 7-day-old Sprague–Dawley rats as previously described [23]. Cells were seeded on poly-L-lysine-coated dishes at a density of 250,000 cells/cm^2 and cultured in basal medium Eagle's (BME medium) supplemented with 10% inactivated fetal calf serum, 25 m*M* KCl, 0.5% (v/v) penicillin–streptomycin. Cytosine arabinoside (10 μM) was added to the cultures 48 h after seeding to prevent growth of glial cells. CGC were used for experiments after 8–9 days. At this time, the cultures contained over 95% neuronal cells [24].

Viability assay. Cell viability was measured by the MTT tetrazolium salt assay [25]. This colorimetric assay is based on the capacity of mitochondrial enzymes in viable cells to transform the MTT tetrazolium salt into MTT formazan, which can be detected by spectrophotometry. This assay also reflects the capacity of electron transferring reactions in the mitochondrial respiratory chain [26]. For these determinations, cells were plated in 24-well plates at a density of 250,000 cells/cm^2/well in a volume of 0.5 ml. MTT tetrazolium salt was dissolved in serum-free culture medium to a final concentration of 0.3 mg/ml and added to the cells for 1 h at 37°C. The medium was then removed, isopropanol (99.9%; v/v) was added, and the absorbance was detected at λ = 592 nm on a Multiscan reader.

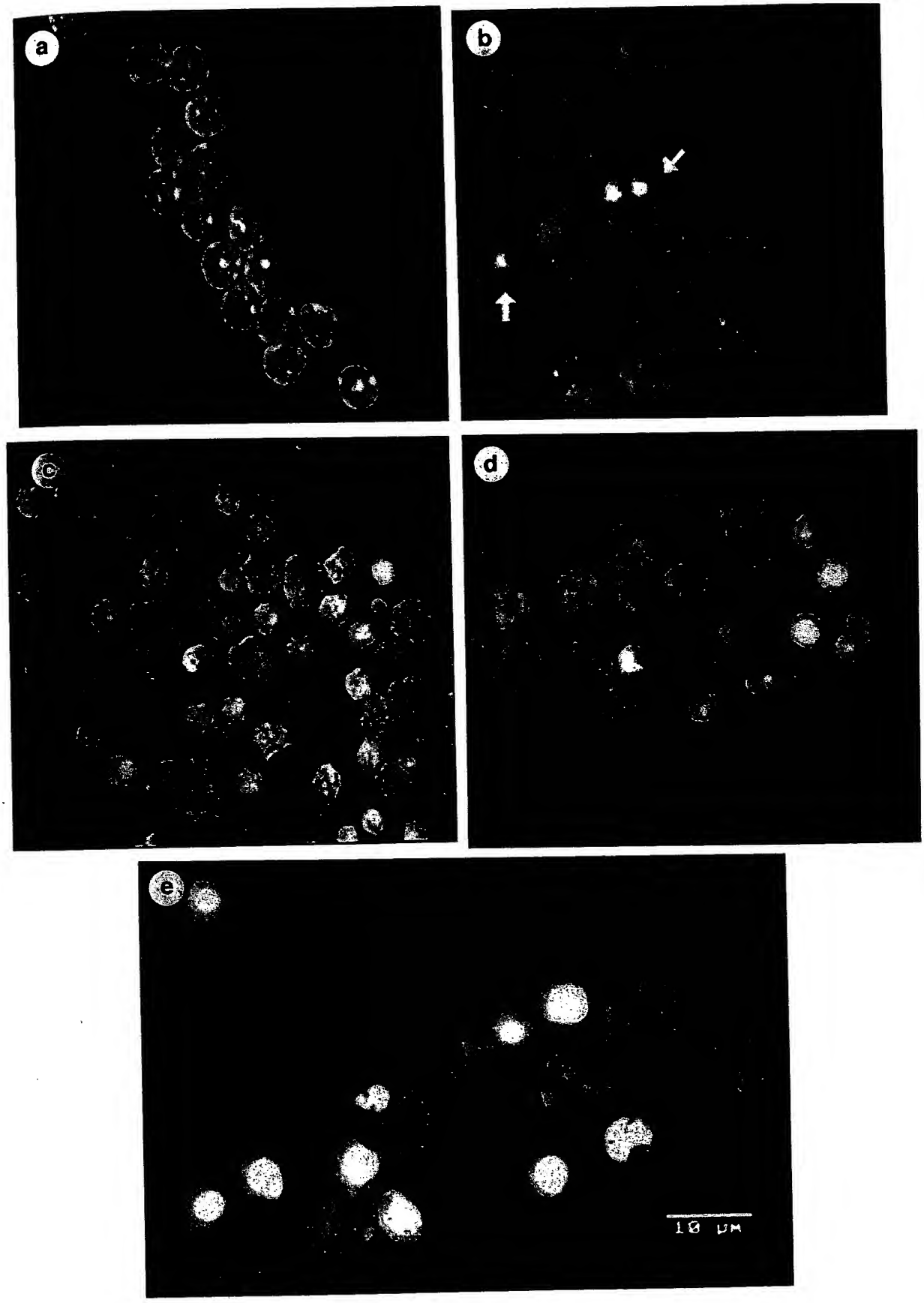
Detection of apoptotic cells. CGC were cultivated and exposed as described above on poly-L-lysine-coated coverslips. After exposure, cells were fixed in methanol:water (80:20) for 10 min, washed in PBS, and subsequently stained with propidium iodide (5 μg/ml) for 5 min. Coverslips were mounted onto glass slides in glycerol:HBS (1:1) and examined in a Bio-Rad MRC 600 confocal microscopy system using the 488-nm excitation line of the krypton/argon laser. Nuclei of untreated cells revealed a typical chromatin morphology with distinct organization, whereas apoptotic nuclei were highly fluorescent, condensed, and displayed polarized chromatin aggregates. Apoptotic cells were scored and 10 fields or more than 400 cells were counted for each sample from three different experiments.

DNA fragmentation. Cells were grown on 100-mm Costar dishes. After treatment with colchicine and subsequent incubation for 12, 18, and 24 h, cells were removed, lysed, and centrifuged to remove intact (pellet) from fragmented (supernatant) DNA. Supernatants were extracted with phenol and chlorophorm:isoamylalcohol (24:1) as previously reported [27]. Agarose gel electrophoresis was performed according to Wyllie [28], with minor modifications. Samples were loaded on 1.2% agarose gels and electrophoresis was performed applying 60 mA current. DNA was visualized under uv light (305 nm) after staining with ethidium bromide (6 μg/ml).

$[Ca^{2+}]_i$ determinations. Fluorescence measurements of $[Ca^{2+}]_i$ in cells monolayers attached to coverslips were made using a SPEX Fluorolog-2 system. The coverslip formed the bottom of an open perfusion chamber (volume: 100–150 μl) connected to a two-channel peristaltic pump (Ismatec) allowing a constant perfusion of the cells, with a flow rate of 0.3 ml/min. The chamber was mounted on a thermostatically controlled stage of an inverted microscope (Zeiss Axiovert 135M). The microscope was coupled to a SPEX Fluorolog-2 system providing excitation with the wavelength pair 350/380. Cells on coverslips were loaded with 2 μM fura-2/AM for 30 min at 37°C. Coverslips were washed free of fura-2 and fluorescence and changes were measured as a ratio of excitation wavelengths 340/380 with the emission set at 510 nm [29]. $[Ca^{2+}]_i$ was calculated according the formula: $[Ca^{2+}]_i = K_d \cdot (R - R_{min})/(R_{max} - R) \cdot (sf_2/sb_2)$, where K_d was assumed to be 224 nm at 37°C and R was the experimentally determined ratio. R_{max} is the maximal ratio obtained using the Ca^{2+} ionophore, ionomycin, to saturate the dye with Ca^{2+}. $MnCl_2$ was used to quench the dye and obtain the minimum ratio (R_{min}). sf_2/sb_2 is the ratio of fura-2 fluorescence value at 380 nm for free and Ca^{2+}-bound dye, respectively [29]. The contribution of extracellular fura-2/AM was subtracted after quenching the fluorescence with 50 μM $MnCl_2$.

Immunocytochemistry. After exposure, cultures were rinsed in PBS, fixed, and permeabilized in methanol:water (80:20) for 10 min, followed by treatment with acetone for 10 min at room temperature. For cytoskeletal staining, we used primary antibodies, a mouse monoclonal antibody raised against the tau protein and a rabbit polyclonal antibody against the α and β tubulines used at 1:200 dilution. Coverslips were incubated with the antibody for 30 min, rinsed in PBS, and stained with secondary antibodies, anti-mouse IgG-fluorescein, or anti-rabbit IgG fluorescein F(ab)$_2$ fragment (Boehringer-Mannheim) (dilution 1:64) for 30 min. Coverslips were wet-mounted in glycerol and imaged with a Bio-Rad MRC-600 confocal microscope using an oil-immersion, Nikon 60X plan-apo lens (n.a. 1.4). A similar procedure was used to visualize c-Fos and NOS (Abbott Laboratories). Cells were stained with the first antibody overnight at 4°C and then rinsed in a PBS supplemented with 2% BSA, 0.2% Tween 20, and 1 m*M* sodium azide. Coverslips were exposed to the secondary antibody, an anti-rabbit IgG–fluoresceine conjugate for 30 min at room temperature. In some experiments, chromatin was stained with 5 μg/ml propidium iodide for 5 min. Controls included the substitution of nonimmune serum for the primary antibody and omission of the primary antibody. Coverslips were mounted and imaged as described above ex-

FIG. 1. Apoptotic nuclei in CGC treated with colchicine. CGC on glass coverslips were exposed to 1 μM colchicine and at the time indicated fixed, permeabilized, and stained with propidium iodide as described under Materials and Methods. (a) Untreated cells at 12 h. (b) Colchicine 12 h: at the confocal microscope, a few apoptotic nuclei are visible (shown by the arrows). They appear highly fluorescent, typically pyknotic, without discernible chromatin structure. Their average size was 2.26 ± 1.8 μm, whereas control nuclei had a diameter of 4.1 ± 2.2 μm. Nuclear diameters were calculated using the Bio-Rad software Comos. (c) CGC treated with colchicine for 18 h. (d and e) Colchicine-treated cells at 24 h. In e a high-magnification detail is shown.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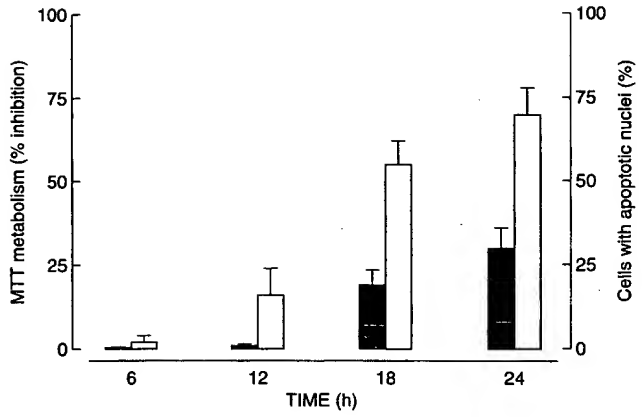


FIG. 2. Apoptotic bodies and MTT metabolism in colchicine-treated CGC. Apoptotic bodies (□) and the ability of CGC to metabolize MTT (■, ▨) were measured in cells treated with colchicine alone (■) or with colchicine plus 100 n*M* taxol (□). Apoptotic bodies were scored in 10 fields for each sample/time point and in each of three separate experiments. Results represent means ± SD of three (□) or four (■) different experiments.

cept that a dual-wavelength setup was used to collect the antibody fluorescence (green) and the nuclear staining (red).

Nitrite (NO_2^-) determination. NO_2^- levels were measured by a procedure based on the Greiss reaction as described by Green [30]. Aliquots of 50 μl medium were taken from the culture plates and used as a background for NO_2^- production. The Greiss reagent (50 μl) was added to each sample in 96-well plates. Plates were let at room temperature for 10 min and the absorbance at λ = 546 nm was detected in a Microscan reader.

RESULTS

Colchicine Causes CGC Apoptosis

CGC underwent apoptosis between 12 and 24 h after the addition of colchicine (Fig. 1). Nucleic acid staining with propidium iodide revealed typical apoptotic nuclei, which exhibited highly fluorescent condensed chromatin without any discernible structure. CGC are normally small, characterized by a predominant nucleus surrounded by a cytoplasmic rim. In nonapoptotic cells the nuclear size was about 4.0 μm, whereas it was reduced to 2.0–2.4 μm in apoptotic cells (see Fig. 1). Notably, apoptotic cells did not lose adherence to the dishes and maintained the dendritic network, as recently observed in other neural cell types [31]. The number of apoptotic bodies increased significantly between 12 and 24 h (Fig. 2). Mitochondrial MTT metabolism decreased only at a later stage (i.e., after 18 h), showing that mitochondrial function and cell integrity were preserved in CGC undergoing apoptosis. Even after 24 h, MTT metabolism was inhibited in only 30%, while over 70% of the cells displayed apoptotic bodies (Fig. 2).

Although DNA "laddering" seems to occur as a mid-to-late event in some *in vitro* systems of apoptosis [32, 33], DNA fragmentation by a Ca^{2+}- and Mg^{2+}-dependent endonuclease [28] remains a typical hallmark of apoptosis. In CGC exposed to colchicine, DNA laddering was visible after 18 to 24 h (Fig. 3A). At 12 h, when apoptotic nuclei became visible, bulk DNA aggregates appeared on top of the agarose gels. Such DNA aggregates not resolved by conventional agarose gel electrophoresis corresponded to chromatin fragments of 300 and 50 kbp when analyzed by pulsed-field gel electrophoresis (data not shown), as previously observed in other systems [34]. Pretreatment of CGC with 100 n*M* taxol, a microtubule stabilizing agent, prevented colchicine-induced DNA laddering (Fig. 3A), whereas treatment with NOS inhibitors did not affect DNA fragmentation. Similarly, treatment with Ca^{2+} channel blockers including the L-type antagonists nifedipine and verapamil or the NMDA antagonist MK-801 was without effect (Fig. 3B).

Cytoskeletal Alterations

The neurotoxicity of colchicine results primarily from its ability to bind and modify tubulin, as nontubulin binding analogs are not neurotoxic [35]. We examined the effects of colchicine on the tau protein and α and β tubulins by immunocytochemistry. In untreated cells,

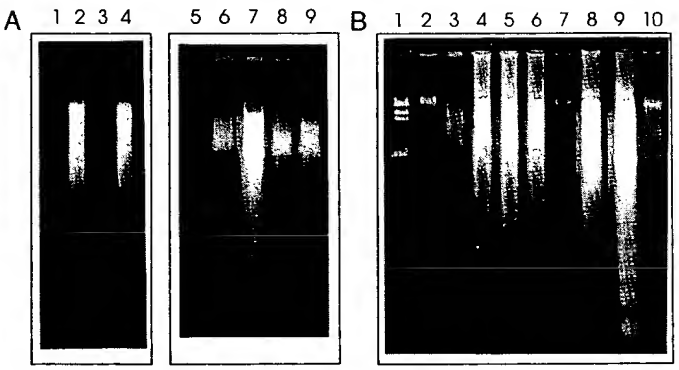


FIG. 3. DNA fragmentation in colchicine-treated CGC. (A) Colchicine induces DNA laddering in CGC at 18 and 24 h after exposure. CGC were treated with 1 μ*M* colchicine. At the time indicated, fragmented DNA was separated from intact DNA, extracted, and loaded onto agarose gels as described under Materials and Methods. Lane 1, CGC control at 18 h. Lane 2, cells treated with 1 μ*M* colchicine for 18 h. Lane 3, CGC control at 24 h. Lane 4, cells treated with 1 μ*M* colchicine for 24 h. At 12 h after colchicine treatment, only large-size chromatin aggregates were visible on top of the gels. Incubation with taxol inhibited DNA laddering, but did not prevent the formation of the large chromatin aggregates. Lane 5, CGC control at 12 h. Lane 6, CGC treated with 1 μ*M* colchicine for 12 h. Lane 7, CGC exposed to colchicine for 18 h. Lanes 8 and 9, CGC pretreated with 100 n*M* taxol and then exposed to 1 μ*M* of colchicine for 18 h (lane 8) or 24 h (lane 9). (B) Effect of various inhibitors. Lane 1, DNA markers: pBR 328 DNA-*BglI*. Lane 2, control. Lane 3, CGC exposed to 1 μ*M* colchicine for 12 h. Lane 4, CGC exposed to colchicine for 18 h. Lane 5, CGC exposed to colchicine for 24 h. Lane 6, CGC treated with 100 μ*M* NMMA and colchicine at 18 h. Lane 7, control treated with NMMA at 18 h. Lane 8, CGC treated with nifedipine and colchicine for 18 h. Lane 9, CGC treated with MK801 and colchicine for 18 h. Lane 10, controls treated with MK-801 at 18 h showed no DNA laddering.

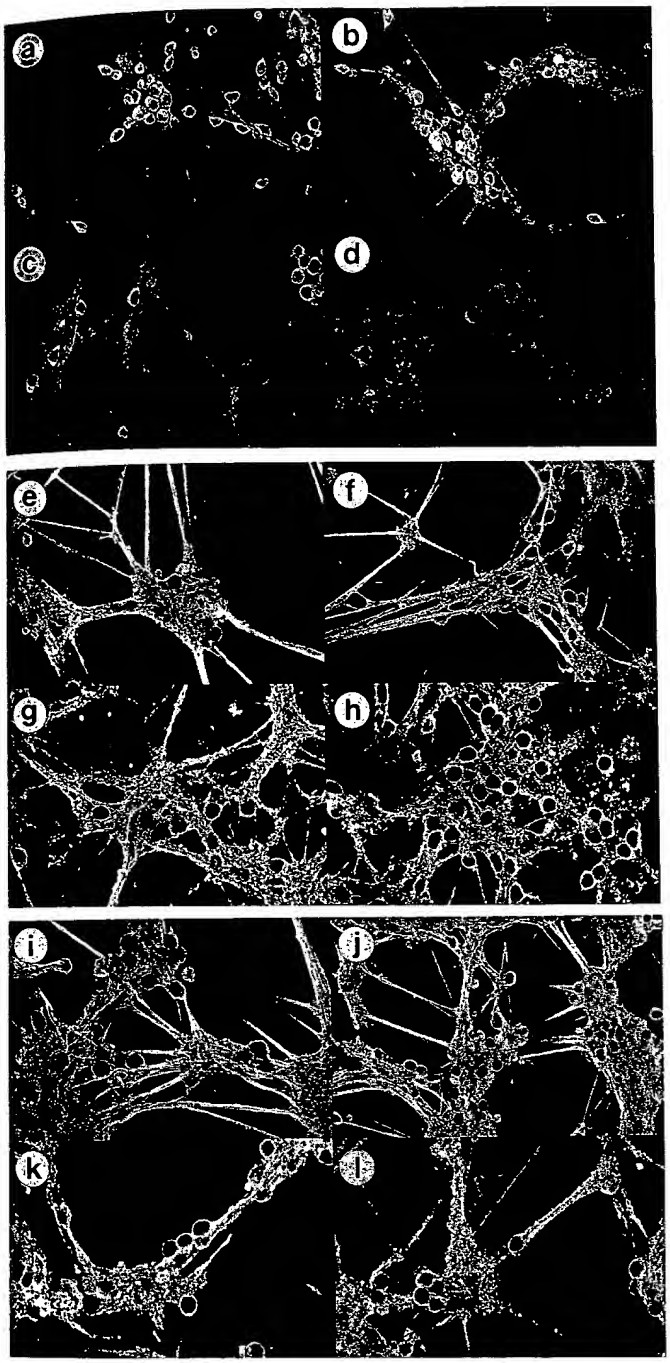


FIG. 4. Tau and α and β tubulin distribution in CGC exposed to colchicine. Tau protein immunostaining: (a) untreated cells at 6 h; (b) untreated cells at 12 h; (c) after colchicine treatment, 6 h; (d) after colchicine treatment 12 h. α tubulin immunostaining: (e) untreated cells at 6 h; (f) untreated cells at 12 h; (g) after colchicine treatment, 6 h; (h) after colchicine treatment 12 h. β tubulin immunostaining: (i) untreated cells at 6 h; (j) untreated cells at 12 h; (k) after colchicine treatment, 6 h; (l) after colchicine treatment 12 h.

tau immunostaining was typically more intense around the cell body, with less intense staining of the axons (Figs. 4a and 4b). A similar distribution around the cell nucleus was visible for the α and β tubulins (Figs. 4e, 4f, 4i, and 4j). However, here, the peripheral staining was more intense, particularly in the area where axons originated from the cell soma. Alterations in the fluorescence pattern of tau protein became apparent 6 h after exposure to 1 μM colchicine (Fig. 4c). Fluorescence intensity was reduced in the axons and in many cell bodies. A residual staining was still localized in the cytoplasmic rim that envelops the CGC nucleus. A generalized loss of tau positive immunostaining was instead evident at 12 h, when the fluorescence in the soma was also markedly reduced (Fig. 4d). The assembly of α and β tubulins was also disturbed. Initial fragmentation—visible as loss of continuity and granular staining—was initially appreciable in the axons at 6 h and clearly visible at 12 h (Figs. 4g, 4h, 4k, and 4l).

Alterations of Ca^{2+} Responses to KCl Depolarization

To investigate whether colchicine treatment influenced the Ca^{2+} response to high K^+, fluo-3-loaded CGC were examined by confocal microscopy. Typical individual CGC responses to high K^+ are illustrated in Fig. 5A. After treatment with colchicine (i.e., beginning at 2 h), Ca^{2+} responses to KCl depolarization were enhanced. The peak Ca^{2+} response to KCl depolarization increased 2 h after treatment with colchicine. The potentiation was evident in most cells and was followed by a prolonged recovery phase (Fig. 5A, traces in b). After 4 h the recovery was most definitely slow and incomplete (i.e., Ca^{2+} did not return to resting levels; traces in c). To quantify the variation in Ca^{2+} responses to depolarization due to colchicine treatment, cells were loaded with the two-wavelength dye, fura 2, which allows ratiometric measurements of Ca^{2+} concentration, and imaged by conventional fluorescence microscopy (Fig. 5B). Similar to what observed with fluo-3 in the confocal measurements, the peak Ca^{2+} response to depolarization increased 2 h after the treatment with colchicine (not shown), and by 4 h a prolonged and incomplete recovery was evident. The intracellular Ca^{2+} changes were quantified over time. As shown in Fig. 5C, the Ca^{2+} increase elicited by depolarization at 4 h was twice as high in colchicine-treated cells compared to control cells identically stimulated. Colchicine treatment also promoted an increase in the resting Ca^{2+} level. A moderate but sustained Ca^{2+} increase appeared after 2 h and was maintained up to 6 h. This increase was sensitive to pretreatment with L-type channel blockers such as nifedipine and verapamil, but not to the NMDA blocker, MK-801 (data not shown).

C-Fos Expression in Apoptotic CGC

Studies in the *fos–lacZ* transgenic mouse have recently shown that c-Fos induction precedes cell pyknosis and deletion in naturally occurring cell death and also in the neurodegeneration caused by kainic

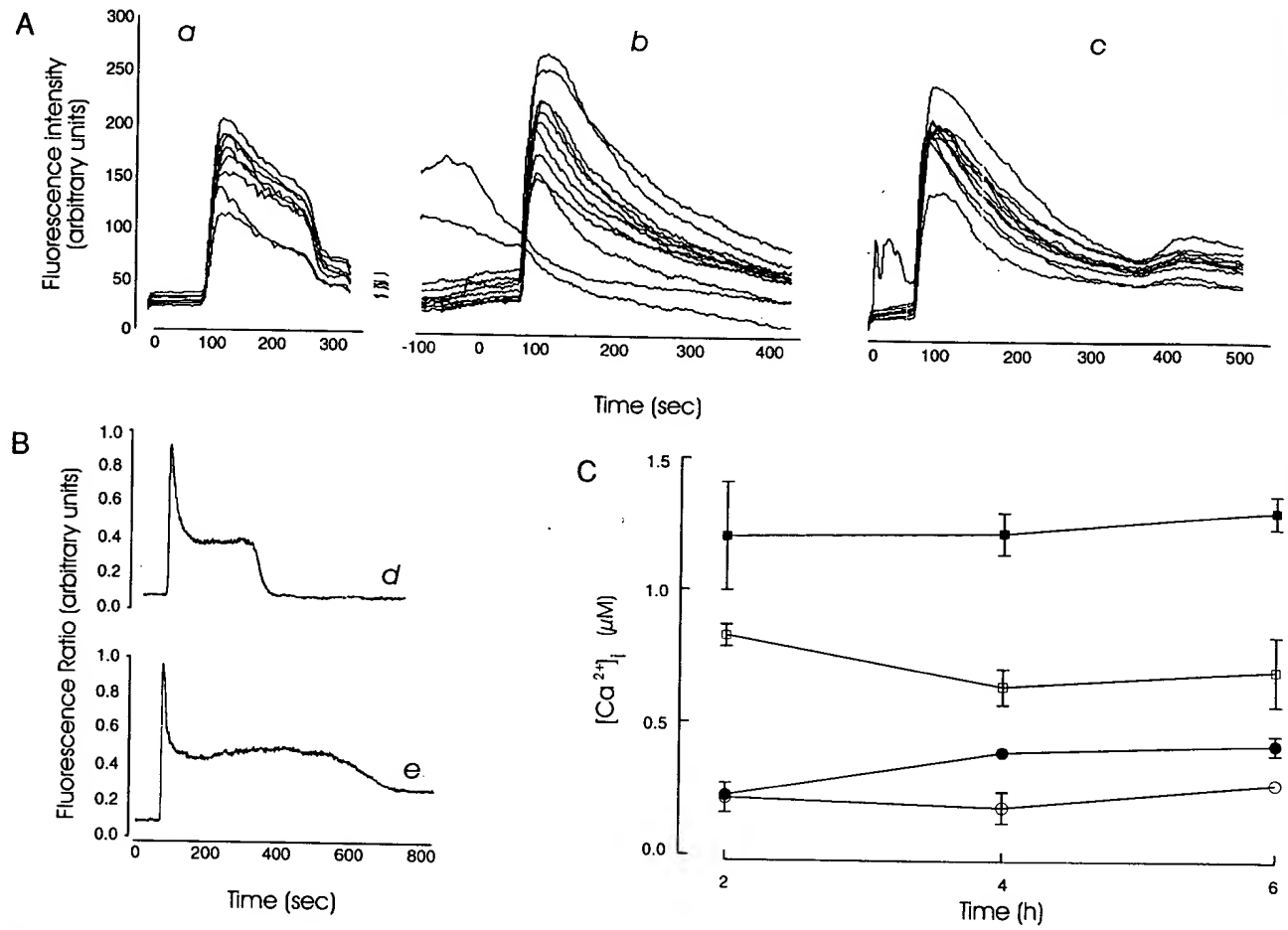


FIG. 5. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) in individual cells treated with colchicine and stimulated by chemical depolarization with KCl. (A) $[Ca^{2+}]_i$ was measured in fluo-3-loaded CGC as described under Materials and Methods. (a) Untreated cells were stimulated with 50 m*M* KCl. (b) Cells were exposed to colchicine for 2 h and stimulated as indicated in a. The average peak $[Ca^{2+}]_i$ response was enhanced and the plateau phase was replaced by a slow decline; 4 h after colchicine treatment the recovery was followed by a secondary $[Ca^{2+}]_i$ increase in some cells, whereas in nearly all cells $[Ca^{2+}]_i$ did not return to the resting level. The single traces represent individual cells. (B) Cells were loaded with fura-2/AM as described under Materials and Methods, and groups of cells were imaged using a SPEX Fluorolog system; the average fluorescence ratios obtained from a group of 4–6 cells are illustrated. Traces represent typical recordings from seven separate experiments, where at least 20 to 30 cells/coverslip were imaged. Trace d, untreated cells; trace e, colchicine 4 h. (C) The fluorescence ratios obtained by the measurements illustrated in B were calibrated as indicated under Materials and Methods and values of $[Ca^{2+}]_i$ are displayed as function of time of exposure to colchicine. (●, ○) Resting $[Ca^{2+}]_i$; (■, □) peak $[Ca^{2+}]_i$ after stimulation with 50 m*M* KCl. Filled symbols refer to cells treated with 1 μM colchicine.

acid [36]. Further, it was shown that c-Fos expression colocalizes with the areas where cerebellar granule neurons degenerate in the *weaver* mouse [36]. These observations prompted us to investigate whether c-Fos expression would occur before apoptosis induced by colchicine in cerebellar granule cell cultures. Between 4 and 6 h after colchicine addition c-Fos expression was observed in most cells typically confined to the nucleus (Fig. 6a), whereas c-Fos levels were undetectable in untreated cells. This effect was transient, and in most neurons c-Fos immunostaining decreased by 12 h. However, some cells, apparently preapoptotic and undergoing chromatin condensation, retained a positive c-Fos immunostaining (Fig. 6b). In these cells, confocal microscopy revealed a rather unusual c-Fos localization (Fig. 6c). While the protein was concentrated in the nuclei presenting chromatin margination, positive staining was found also in the cytoplasm. It appeared as if in apoptotic cells part of the c-Fos gene product was excluded from the nuclei with condensing chromatin. In some neurons, where the nucleus had finally become pyknotic and the chromatin was condensed and marginated, Fos immunoreactivity was retained, typically separated from the residual chromatin aggregates (Fig. 6d).

Nitrate Production and NOS Expression in Colchicine-Treated Cells

One response to increased Ca^{2+} stimulation in neural cells involves the activation of a constitutive form of the

nitric oxide synthesis (NOS) that promotes NO production [37]. The role of different chemical forms of NO is still controversial, although clearly in several neurophatological conditions NO production has adverse effects on cell survival [38, 39]. In colchicine-treated cells, NO production was not significantly modified at 6 h, but steadily increased after 12 h (Table 1). The increase was prevented by a NOS inhibitor, NMMA (Table 1). Since in colchicine-treated cells at 6 h, the resting Ca^{2+} level was already higher than that of control cells, we could not attribute the increased NO production to the alteration in intracellular Ca^{2+}. Experiments were therefore performed to investigate whether the increased NO production was associated with an increased NOS expression. Immunocytochemical studies showed that NOS distribution was consistent with a cytosolic localization in the rim around the nucleus and primarily along the axons (Figs. 7a and 7c). The intensity of NOS staining increased clearly at 12 h (Figs. 6b, and 6d), when cells began to show the chromatin modifications typical of apoptosis.

Effect of Various Agents on Colchicine-Induced Apoptosis

In view of the findings described above, several agents were used in the attempt to gain insight to the most decisive event leading to CGC apoptosis after colchicine stimulation. As shown in Table 2, taxol, a microtubule stabilizing agent protected CGC from colchicine-induced cell killing. MK-801, an inhibitor of NMDA receptors or L-type Ca^{2+} channel blockers, had instead no effect on colchicine-induced cell death. Inhibitors of the NOS such as NMMA or nitroarginine (not shown) exerted a protective effect although to a lesser extent. Interestingly, cyclosporin A, which protects mitochondria from excess Ca^{2+} cycling, also protected from the loss of viability induced by colchicine. However, except taxol, none of the agents mentioned above could prevent apoptotic body formation and DNA laddering. Thus, it appears that the primary cytoskeletal alterations caused by colchicine were sufficient to trigger both apoptosis and the secondary cell lysis. In contrast, inhibiting NO production or using cyclosporin to block mitochondrial Ca^{2+} cycling prevented only the postapoptotic events that resulted in cell disintegration.

DISCUSSION

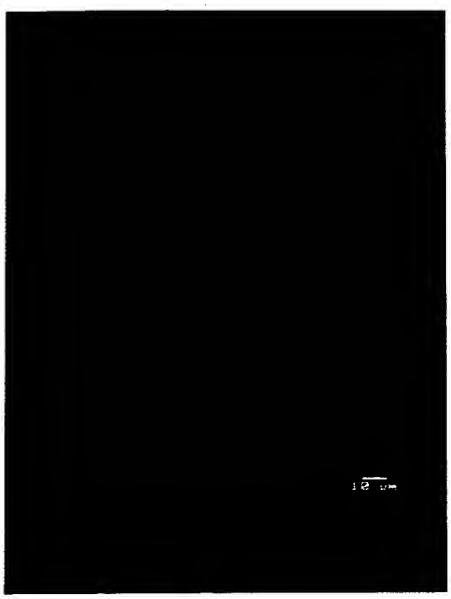
The cytoskeleton undergoes significant changes during apoptosis. Blebbing, cell shrinkage, and chromatin condensation are associated—in some systems—with alterations in microfilament proteins [40] and a disruption of nuclear lamins [41]. The role of cytoskeletal alterations in the development of apoptosis is unknown. In particular, it is unclear whether such alterations are an unavoidable implication of cell volume changes and nuclear pyknosis, or whether these result from early modifications of cytoskeletal elements. Disturbances of the cytoskeletal organization could be caused by other events including modifications in protein phosphorylation/dephosphorylation, Ca^{2+} overload, protease activation, or protein–protein cross-linking [42].

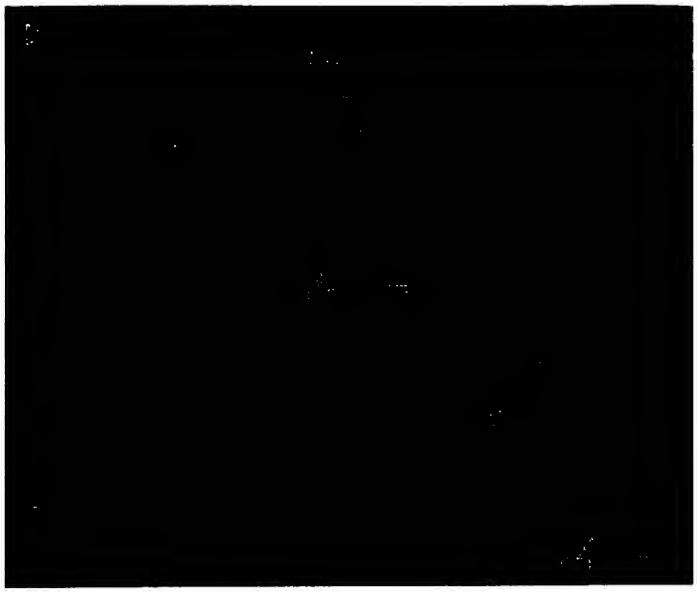
The results of this study show that a initial cytoskeletal damage such as that caused by a microtubule disrupting agent is sufficient to trigger apoptosis in primary neural cell cultures. The finding that—after colchicine treatment—taxol protected CGC from undergoing apoptosis supports this idea. Agents which disrupt microfilaments can also induce apoptosis in other cell types and recently, it has been reported that colchicine can induce apoptosis in liver cells [43]. Furthermore, alterations in tau phosphorylation, because of β-amyloid-induced activation of tau protein kinase I, may initiate active cell death in cells of neural derivation [11].

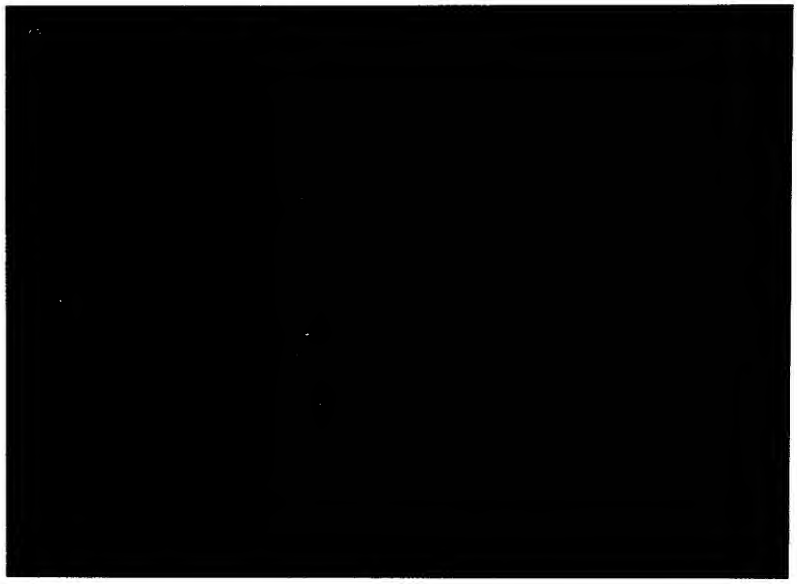
The relatively slow development (2–6 h) of visible cytoskeletal modifications and the other adverse effects evoked by colchicine are consistent with the property of this drug to bind tubulin with very high affinity, but rather low kinetics [44]. Microtubules are dynamic structures whose organization is strictly controlled by the equilibrium between tubulin heterodimers and assembled tubulin. The exchange rate between these two pools is very rapid (about 10 min in quiescent cells and 30 s in mitotic cells) [45]. This makes microtubules very efficient in remodeling to respond to changes in the intracellular environment, but renders the microtubular system quite vulnerable to agents that bind to tubulin [46]. Colchicine can suppress microtubule dynamics and promote their disassembly, because of the low amount of tubulin-colchicine that becomes incorporated into the microtubules [47]. On the contrary, taxol promotes microtubule assembly [48] and stabilization [49], counteracting the effect of colchicine [50]. Notably, taxol can also reduce neural immunoreactivity toward Alz-50 and 5E2 in cultures exposed to glutamate or Ca^{2+} ionophore [9].

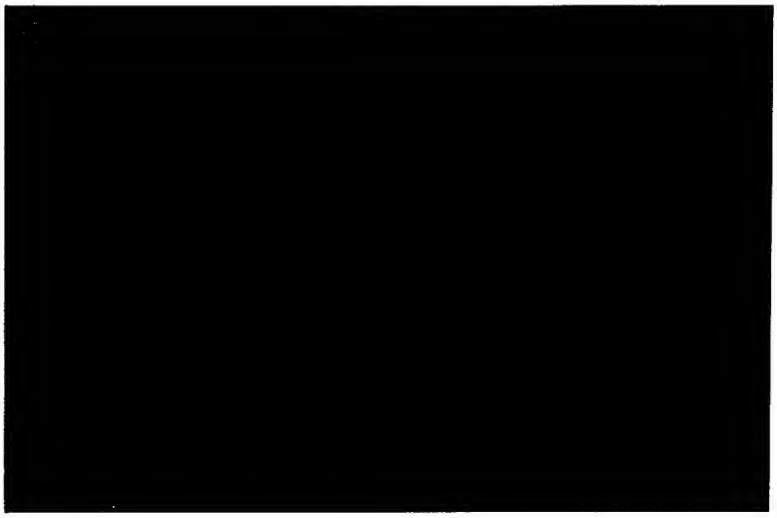
Intracellular Ca^{2+} accumulation and overwhelming generation of NO have both been implicated in neural

FIG. 6. c-Fos immunostaining after colchicine treatment. (a) c-Fos immunostaining (green) 6 h after colchicine treatment. (b) At 12 h, c-Fos immunostaining was positive in a few neurons, some of which appeared preapoptotic (i.e., smaller size and nuclear margination). Few apoptotic cells could also be seen as small intensively red-stained bodies. (c) Localization of c-Fos immunostaining in these cells was abnormal. At high magnification (a 3.3× zoom setting was selected on the confocal using a 60X plan-apo lens 1.4 n.a.) the protein was retained only in the center of the condensing nuclei, while the chromatin marginated at the nuclear periphery. Positive staining was also evident outside the nucleus, suggesting either a loss of protein from the nucleus or a block of protein import in the nuclear matrix. Finally, in d, some nuclei with residual chromatin fragments retained Fos immunostaining. Images are typical of four separate experiments in which 10–15 fields were imaged or more than 500 cells.









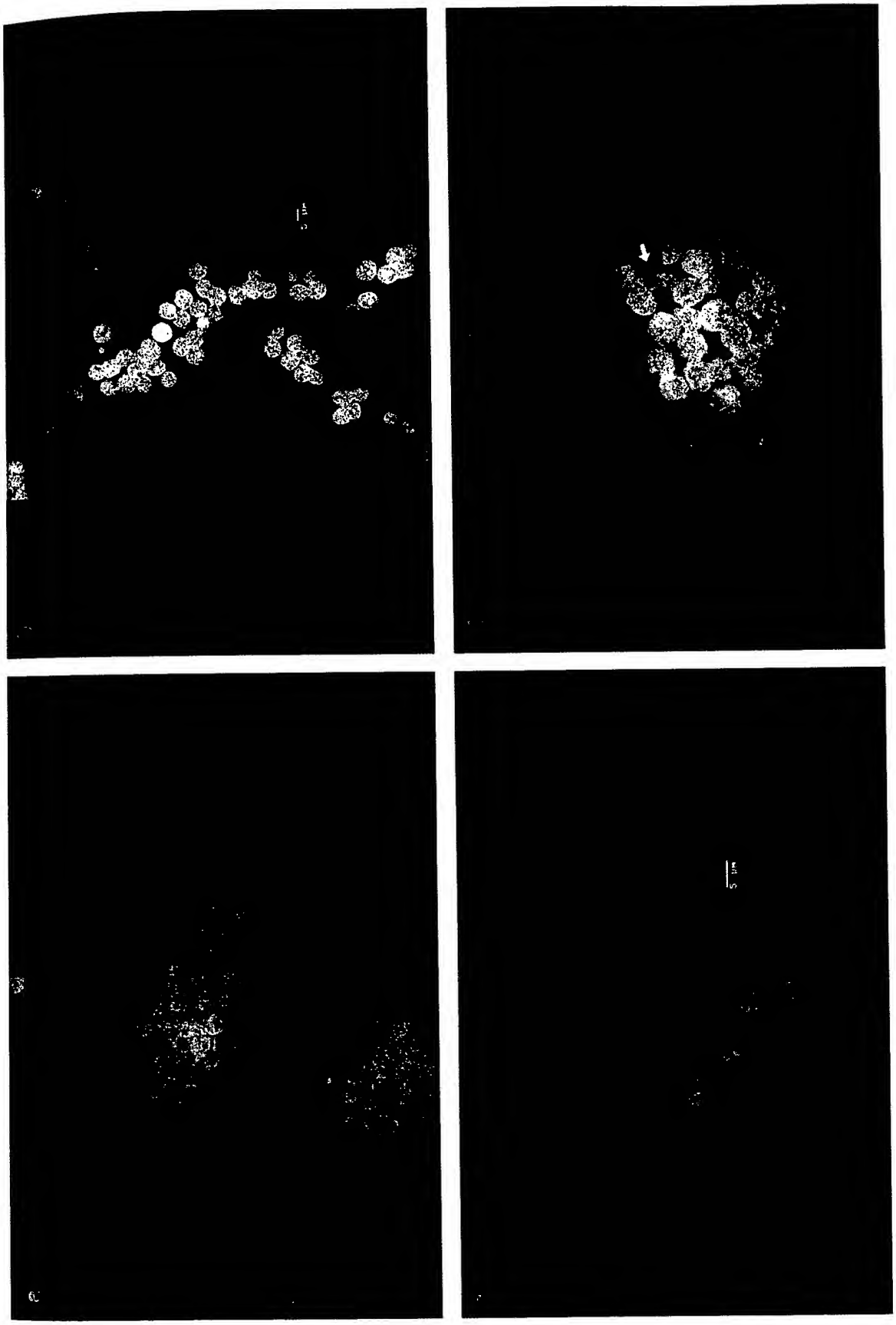


FIG. 7. NOS immunostaining. Cells were treated with colchicine as described under Materials and Methods. In untreated cells or in cells treated with colchicine for 4 or 6 h NOS staining was visible in the the cell bodies (a) and in the projections (c). In some cells, positive staining was visible primarily in the projections and in the cytoplasmic rim around the nucleus (c). After a 12-h treatment with colchicine, the staining had intensified in both the periphery and around the cell body (b, d). Images were collected keeping a constant gain value and black level. A semiquantitative analysis of the images using the Bio-Rad Comos software revealed an increase in the staining intensity of about 30% in most areas. At least 30 cells were analyzed per field and 10 separate fields per sample were considered. Samples were collected from three separate experiments. Images in c and d were collected using 2.5× zoom.

TABLE 1

Production of NO in CGC Exposed to Colchicine

Treatment	$\mu M\ NO_2^- \times 10^{-6}$ cells
Control	8.7 ± 1.0
Colchicine 6 h	8.8 ± 0.9
Colchicine 6 h + NMMA	8.0 ± 0.6
Colchicine 12 h	10.8 ± 1.3
Colchicine 12 h + NMMA	7.9 ± 1.4
Colchicine 18 h	12.4 ± 1.7
Colchicine 18 h + NMMA	8.2 ± 1.1

Note. NO production was measured as described under Materials and Methods. Results represent the mean ± SD from three separate experiments.

cell death. Thus, we investigated as to whether such mechanisms would mediate colchicine-induced CGC apoptosis. Evidently cytoskeletal elements can control both Na^+ and Ca^{2+} channel function [16, 18]. Thus, while colchicine seems to speed Ca^{2+} channel decline in Lymnea neurons, taxol and phalloidin were found to prolong Ca^{2+} channel activity [18]. The effects observed in our study (i.e., increased transient Ca^{2+} peak and delayed recovery) are congruous with the conclusion that colchicine treatment results in a prolonged channel activity in CGC. At present, we do not know the mechanism responsible for this effect. While a detailed study of Na^+ or Ca^{2+} channel properties in CGC is outside the scope of this work, we can speculate that an allosteric interaction between cytoskeletal elements and channels would modulate Ca^{2+} effects on Ca^{2+} channels as suggested by other studies [16, 51]. Since NMDA antagonists (i.e., MK-801) did not affect colchicine-induced modification of Ca^{2+} responses to high K^+, and colchicine does not alter intracellular levels of Cl^-, K^+, and Na^+ in other systems [17], we suggest that the observed effects are limited to voltage-operated Ca^{2+} channels, possibly of the L-type.

Nevertheless, the findings that neither NMDA nor L-type Ca^{2+} channel blockers prevented apoptosis suggest that the Ca^{2+} channel alterations induced by colchicine may not be sufficient to activate the process in this system. It is possible that the amplitude of the Ca^{2+} accumulation may be critical and that moderate increases such as that observed after colchicine treatment are not sufficient to trigger the cell death program. On the other hand, phosphorylation of cytoskeletal elements (i.e., vimentin) is known to promote gene exposure and to regulate transcription [52]. Thus, a direct alteration of the nucleoskeleton may contribute to promote initial chromatin alterations that would facilitate Ca^{2+}-mediated DNA cleavage and nuclear condensation.

An increased gene exposure because of microtubule disruption, with higher resting Ca^{2+} levels, may also be responsible for the increased expression of c-Fos. Colchicine is indeed known to induce c-Fos mRNA and other mRNAs following *in vivo* injection [53]. Although c-Fos expression may simply be an ancillary phenomenon in neural cell apoptosis as other stress responses, it is noteworthy that the c-Fos protein remains expressed in cells undergoing apoptosis and is characteristically excluded from preapoptotic nuclei (i.e., from marginated nuclei with an intact envelope, initial chromatin rearrangement in apparently normal cells). Similar findings have been reported in recent *in vivo* studies [36].

Another protein overexpressed before CGC apoptosis was NOS. We decided to study NOS expression when experiments aimed to measure NO production revealed that NO levels increased only 12 h after colchicine treatment. Therefore, the rise in the resting Ca^{2+} levels observed earlier could not explain the stimulation of NO production in colchicine-treated cells. Similar induction of the neuronal NOS has been recently observed after peripheral axotomy [54]. The significance of the increased NOS immunostaining in CGC undergoing apoptosis is yet unclear, as remains the role that NO overproduction has in the formation of apoptotic nuclei and the process of DNA fragmentation in this system. The increased NO production in CGC exposed to colchicine was not apparently essential for chromatin condensation and fragmentation, whereas it was critical for the development of CGC secondary necrosis (i.e., plasma membrane damage and loss of mitochondrial function). On the other hand, in different neural [55] and nonneural [27] cells, NO overproduction causes all the typical features of apoptosis. This suggests that NO accumulation may elicit either type of cell death, perhaps depending on the exposure level and the cell type or the prevalent intracellular target. It is still unclear whether in CGC exposed to colchicine the NO effect is directed to the DNA, to the mitochondria, or to other intracellular targets. Previous studies in nonneural cells undergoing

TABLE 2

Effect of Different Treatments on Colchicine-Induced Loss of CGC Viability

Treatment	18 h	24 h
Control	100	100
Colchicine (1 μM)	74 ± 2.8	67 ± 2.6
Taxol 100 nM	97 ± 0.3	93 ± 0.3
Taxol + colchicine	90 ± 4.5*	89 ± 2.0*
NMMA (100 μM)	97 ± 1.3	97 ± 1.5
NMMA + colchicine	88 ± 1.3*	83 ± 2.1*
Cyclosporin A (10 nM)	90 ± 3.2	95 ± 2.5
Cyclosporin A + colchicine	89 ± 3.0*	86 ± 1.0*
MK 801 200 nM	99 ± 0.8	99 ± 1.0
MK 801 + colchicine	76 ± 1.5	63 ± 2.0

Note. CGC were treated with 1 μM colchicine and viability was measured as ability to metabolize MTT to formazan. Results represent the mean ± SD from three separate experiments. Statistic analyses were performed by the Student's t test. Cells treated with various inhibitors were compared to those treated with colchicine alone (* $P \leq 0.05$).

apoptosis because of NO overproduction have shown that the tumor suppressor gene product is overexpressed before chromatin degradation [56]. This has suggested a link between NO-mediated DNA damage and apoptosis. Such mechanisms may not be active in CGC exposed to colchicine, as DNA laddering and formation of apoptotic nuclei were not prevented by NOS inhibitors.

Recent studies have also shown that NO generation can cause mitochondrial Ca^{2+} release as result of a direct effect on the mitochondrial membrane potential [57, 58]. Thus, one distinct possibility is that overwhelming NO production may ultimately cause a collapse of the mitochondrial membrane potential, Ca^{2+} release and necrosis. Studies in our laboratory have indeed shown that energy levels [59] and mitochondrial membrane potential [15] are preserved during apoptosis, whereas a loss of membrane potential and mitochondrial function occurs during necrosis in the same cell systems. The observation that mitochondrial failure (as shown by the inhibition of MTT metabolism) was unrelated to the onset of chromatin condensation and DNA fragmentation, conforms to the idea that cell energy level is maintained during the early phases of apoptosis. Collapse of mitochondrial membrane potential and energy deprivation would instead be invariably associated with secondary necrosis. The observation that cyclosporin A did not prevent apoptosis, but delayed the secondary necrosis, supports this assumption.

In conclusion, our studies show that colchicine-induced cytoskeletal alterations can result in neural cell apoptosis. Postmitotic neurons can undergo active cell death when exposed to a variety of stimuli that can potentially cause cytoskeletal alterations including oxidative stress, glutamate-induced Ca^{2+} overload, or exposure to neurotoxicants. Thus, the possibility that cytoskeletal alterations play a relevant role in the development of apoptosis in neurodegenerative diseases deserves further consideration.

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